Antibiotic Selection Pressure Determination through Sequence-Based Metagenomics

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The human gut forms a dynamic reservoir of antibiotic resistance genes (ARGs). Treatment with antimicrobial agents has a significant impact on the intestinal resistome and leads to enhanced horizontal transfer and selection of resistance. We have monitored the development of intestinal ARGs over a 6-day course of ciprofloxacin (Cp) treatment in two healthy individuals by using sequenced-based metagenomics and different ARG quantification methods. Fixed- and random-effect models were applied to determine the change in ARG abundance per defined daily dose of Cp as an expression of the respective selection pressure. Among various shifts in the composition of the intestinal resistome, we found in one individual a strong positive selection for class D beta-lactamases which were partly located on a mobile genetic element. Furthermore, a trend to a negative selection has been observed with class A beta-lactamases (-2.66 hits per million sample reads/defined daily dose; $P = 0.06$). By 4 weeks after the end of treatment, the composition of ARGs returned toward their initial state but to a different degree in both subjects. We present here a novel analysis algorithm for the determination of antibiotic selection pressure which can be applied in clinical settings to compare therapeutic regimens regarding their effect on the intestinal resistome. This information is of critical importance for clinicians to choose antimicrobial agents with a low selective force on their patients' intestinal ARGs, likely resulting in a diminished spread of resistance and a reduced burden of hospital-acquired infections with multidrug-resistant pathogens.

Antibiotic resistance is at present one of the most serious public health issue across the globe, threatening the achievements of modern medicine. The occurrence of hospital-acquired infections has increased steadily, and this leaves only a limited number of therapeutic options, particularly in immunocompromised patients who are reliant on effective antimicrobial substances for treatment and prevention of infection (1, 2). The human intestinal microflora is known to harbor a vast reservoir of antibiotic resistance genes (ARGs) with geographically variable composition and abundance, as shown in samples from Spain that exhibited a higher relative resistance potential than samples from Denmark or the United States (3, 4). The intestinal resistome is a dynamic and open system which constantly alters due to a transfer of resistance determinants within and between bacterial species via mobile genetic elements, such as transposons, prophages, and plasmids (5). Horizontal transfer of ARGs from the intestinal flora to pathogenic microorganisms poses a notable danger. This process is enhanced by treatment with broad-spectrum antibiotics, which also have an impact on nontargeted organisms like commensals, and it presumably enriches the pool of resistance genes accessible to pathogens (6–9). Furthermore, antibiotic treatment might enable already resistant pathogens to gain dominance over other bacterial species, leading to an increased risk of infection with the resistant strain (10). This indicates the relevance of a customized administration of antimicrobial agents, particularly in a hospital environment due to a possibly massive spread of resistance.

Antibiotic stewardship programs (ABS) are one way to improve antibiotic usage in hospitals (11). However, ABS strategies should be based on a detailed understanding of the impact of various antimicrobial agents on the human intestinal resistome in different patient populations and hospital settings. This would allow a more efficient allocation of resources and enable correct decision making about which antibiotics should be used primarily

or restricted. Most of our knowledge about the association of antibiotic selection pressure and resistance spread is derived from studies of cultured microorganisms (12, 13), which reflect only a minor fraction of the overall microbial population in the gut. Thus, such studies do not represent the immense diversity and variation of the intestinal resistome. Alternatively, in a sequence-based metagenomics approach, a comprehensive sequence collection, which includes unculturable and rare taxa in addition to the usually culturable pathogens, is generated from microbial DNA of the human feces and mapped to a resistance gene database, allowing a deeper exploration of ARGs (14, 15) and determination of a more representative selection pressure.

In this study, we investigated the impact of a 6-day course of ciprofloxacin administration on the intestinal resistome and microbiota of two healthy volunteers by applying a sequence-based metagenomics time series analysis and different ARG quantification methods. We propose a novel algorithm for the determination of treatment-induced selection pressure in various groups of ARGs and discuss the merit of such an approach for clinical stud-

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ies that aim at identifying antibiotic regimens which impose a low selective force on clinically relevant ARGs and ARG groups.

MATERIALS AND METHODS

Participants and sampling. Two healthy male adult volunteers were recruited in Tuebingen, Germany. Participants were excluded if they had been treated with antibiotics within the previous 12 months, were aged under 18 years, were pregnant, or had a past reaction to fluoroquinolones. Study approval was given by the local ethics committee (662/2013BO1), and written consent was obtained. A 6-day course of ciprofloxacin (Cp) was administered at 500 mg orally twice daily, corresponding to one defined daily dose (DDD) (16). The two participants were asked to document any symptoms during Cp treatment, but none were reported. Stool samples from both participants were collected before treatment (day 0), during treatment (days 1, 3, and 6), and 2 and 28 days after treatment.

Sample collection and screening for resistant bacteria. Stool samples were collected in sterile plastic devices (Nerbe Plus GmbH, Germany) and stored within 30 min at 4°C. Samples were plated within 3 h of collection on selective agars for the detection of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (chromID ESBL; bioMérieux, France), vancomycin-resistant enterococci (chromID VRE; bioMérieux, France), methicillin-resistant *Staphylococcus aureus* (MRSA) (chromID MRSA; bioMérieux, France), and *Pseudomonas aeruginosa* (Cetrimide agar; Becton, Dickinson, Germany). Plates were incubated aerobically at 37°C for 48 h and were inspected for growth at 24 h and 48 h.

DNA extraction and sequencing. DNA extraction was performed within 3 h of sample collection according to the Human Microbiome Project protocol with minor modifications (http://hmpdacc.org/doc/HMP_MOP_Version12_0_072910.pdf, accessed November 2013) and using the PowerSoil DNA isolation kit (MO BIO Laboratories, CA). In brief, subsamples of ~3 g stool were suspended in 7 ml lysis buffer, shaken for 30 s, and centrifuged at 3,000 rpm for 5 min. One thousand microliters of supernatant was transferred into a bead tube (garnet bead tubes; MO BIO Laboratories, CA) and incubated at 65°C for 10 min, followed by an incubation at 95°C for 10 min with shaking at 300 rpm. Three tubes per sample were stored immediately at –80°C prior to further steps. After unfreezing, 500 µl of tube content was transferred into a new garnet tube after removal of 500 µl lysis buffer from the new tube. DNA extraction subsequently was performed according to the manufacturer's instructions. DNA was eluted in 100 µl water (HyClone water, molecular biology grade; GE Healthcare, Germany). DNA quantification was carried out using a Qubit analyzer (Invitrogen, Life Technologies, Singapore) by following standard protocols. Metagenomic shotgun sequencing was performed at GATC Biotech AG (Constance, Germany) on an Illumina HiSeq 2000 platform using a paired-end sequencing approach with a targeted read length of 100 bp and an insert size of 180 bp.

Real-time PCR for detection of *tetQ* and *cblA*. The *tetQ* and *cblA* genes were detected using the primers *tetQ*-fw (ATCTGCTGTTGCCAGTGG), *tetQ*-rv (TGTATGCCTTCCTTTCGGA), *cblA*-fw (CACTTCCCCCTTGC TCAGTGT), and *cblA*-rv (CGTGAAATCCTGGTCGGGAA), purchased from tib-molbiol, Germany. The real-time PCR was performed in a total volume of 20 µl using the SYBR green jump start mix (Sigma-Aldrich, Germany), 10 µM each primer, 25 mM MgCl₂, and 1 ng DNA template. HyClone water (molecular biology grade; GE Healthcare, Germany) was used as a negative control. The following cycling conditions were applied: 95°C for 30 s, and then 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The specificity of the PCR product was confirmed by agarose gel electrophoresis following Sanger sequencing (GATC Biotech AG, Germany).

DNA abundance calculation was based on the quantification cycle, *c*, defined as the number of cycles at which the fluorescence crosses the threshold. It was assumed that the quantity of the DNA target would double every cycle. DNA target quantity, *T*, was calculated as $T = 1/2^c$.

T was normalized to the value of 1 for the sample with the highest value, with all other values as fractions of 1. The same was applied to the metagenomic quantification values relative abundance (RA) and omega

(ω). Normalized values were visually compared in terms of their quantity and progression over time. A high concordance between real-time PCR and sequenced-based metagenomics was assumed when an *a priori* maximum deviation of 0.25 in each time point between all measures of quantification was not exceeded.

Sequence data analysis and database construction. For 12 samples, a total number of 820,599,346 raw reads with a length of 101 bp was generated. Quality control was performed using FastQC (available from <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), with all sequenced libraries passing commonly applied quality measures. Raw reads were trimmed to contain a minimum quality Phred score of 30 using the FASTX-Toolkit (available from http://hannonlab.cshl.edu/fastx_toolkit/index.html). Read pairs were placed into one file before further processing. Reads were mapped against an assembly of the human genome (release CRCh37, downloaded from NCBI) using an in-house Smith-Waterman-based DNA aligner called SASS aligner, and any read that matched human sequence was removed.

The metagenomic assembly of each sample was performed using Ray Meta version 2.3.1 with a K-mer length of 21 (17). MetaGeneMark version 2.8 was applied to predict proteins from the assembled contigs using the default setting (18). In order to generate a comprehensive study protein catalogue (CSPC), all predicted proteins from 12 samples were pooled into one file. Sequences with a length shorter than 140 amino acids (aa) were removed. The threshold of 140 aa is equivalent to the 10th percentile length of NCBI RefSeq bacterial sequences. Redundant sequences were removed using the cluster algorithm of CD-HIT version 4.6 (19). Proteins with an alignment coverage greater than or equal to 90% of the full length of the shorter sequence and with more than 95% sequence similarity were removed as redundancies. NCBI RefSeq bacterial protein sequences were downloaded from the NCBI FTP site (accessed 31 December 2014) and merged with those sequences of the subject-specific database. An in-house DNA-to-amino-acid alignment program, called MALT (available from <http://ab.inf.uni-tuebingen.de/software/malt/>), was used to translate reads in all six reading frames and then to perform a semiglobal alignment against the given database. Sequences were removed from the subject-specific database if they were at least 99% identical to RefSeq proteins, thereby keeping the RefSeq annotation in the merged database. This database subsequently was merged with the comprehensive antibiotic resistance database (CARD; accessed 2 May 2015; <http://arpcard.mcmaster.ca>). Redundant proteins were removed as described above, keeping the CARD annotation. The generation of the CSPC as well as the analytical flowchart are illustrated in Fig. 1.

Taxonomic profiling. Quality-controlled reads were mapped against the NCBI nr database (accessed 31 December 2014) using DIAMOND (20) and imported into MEGAN version 5 for taxonomic assignment (21). Lowest-common-ancestor parameters were used as the default, with the exception of a maximum expected value of 0.001. Phylogenetic parameters of all samples are displayed in Table S1 in the supplemental material. Principal coordinate analysis of species distribution was conducted with the cluster analysis function in MEGAN. Diversity index values (Shannon and Simpson indexes) were determined with MEGAN on the species level. Before comparison, all samples were normalized to an equal number of reads to account for unequal sampling effort. Stream plots were generated by the streamgraph htmlwidget R package, and horizon plots were generated by the lattice extra R package. The horizon scale was not specified by default, and the Δ value was calculated for each phylum by subtracting the lowest from the highest sample read count, followed by division by three (<http://lmdvr.r-forge.r-project.org/figures/figures.html>).

Quantification of antibiotic resistance genes and groups. Antibiotic resistance genes were defined as genes with the potency to directly mediate resistance to an antimicrobial agent. Genes that mediate resistance only in the presence of certain mutations or those with regulatory functions were excluded. This was done because short reads do not always cover the variable gene regions which are involved in mediating resistance. Such

FIG 1 Flowchart of the major analysis steps, including databases and construction of the comprehensive study protein catalogue.

reads cannot be used for metagenomic quantification due to the lack of information on whether they are derived from a wild-type gene or a resistant variant of the gene. Regulatory elements, on the other hand, do not directly mediate antimicrobial resistance and were excluded for this reason.

Three different methods of metagenomic quantification were applied and compared. A best-hit algorithm was used for all methods. Quality-controlled reads were aligned against the CSPC database or CARD using DIAMOND (20) with an either 90% or 100% similarity threshold. Mapped reads were counted once respecting the match with the highest bit score. The maximum E value to report alignments was 0.001.

The calculation of the RA has been reported previously (22). For any sample (S), the copy number of antibiotic resistance gene i is given by $b_i = x_i/L_i$, and the relative abundance of antibiotic resistance gene i is given by

$RA_i = b_i/\sum b$, where RA_i is the relative abundance of antibiotic resistance gene i in sample S , L_i is the length of antibiotic resistance gene i in sample S , b_i is the copy number of the antibiotic resistance gene i in sample S , and $\sum b$ is the sum of the copy number of all detected genes in sample S .

We considered this calculation as a reference standard in our study. However, one drawback is that the results depend on the total number of mapped reads, which might considerably fluctuate between samples. Therefore, we used two alternative metagenomic quantification methods that are independent of mapping fluctuations.

To define the first alternative quantification method, for each sample (S) and each corresponding count (x_i) of reads assigned to an antibiotic gene or group i , we used $\omega_i = (x_i/m) \times 10^6$, where m denotes the number

of reads in sample *S*. This is the normalized hit count, ω_i , for the antibiotic resistance gene *i* in sample *S*. The unit of ω_i is hits per million sample reads (hmr). Gene groups can be quantified in the same way.

The third metagenomic quantification method, $\omega_{C,i}$, for the antibiotic gene *i* in sample *S*, is calculated in the same way. The only difference between the two approaches is the database used for the initial mapping, which is CSPEC for ω_i and CARD for $\omega_{C,i}$.

Calculation of selection pressure. We collected cross-sectional time series data to investigate the amount of ARGs across time and Cp administration. Two techniques usually are used to analyze such data, with both accounting for individual heterogeneity: fixed- and random-effects models. These models can calculate the change in ARGs per defined daily dose of an antibiotic, and coefficients constitute appropriate estimates of the antibiotic selection pressure over the period of treatment. The preferred model was chosen on the basis of the Hausman test (23, 24). Furthermore, models were tested for time-fixed effects and heteroskedasticity, and robust standard errors were calculated when the latter was present. Since random-effects models are based on a high number of assumptions, we have tested whether random effects is appropriate by conducting the Breusch-Pagan Lagrange multiplier (LM) test (25). If LM found significant evidence against the use of a random-effects model, an ordinary least-squares regression was run. Model generation and calculation was carried out using Stata version 12.0 (Stat Corp., College Station, TX). A detailed flowchart of the model identification algorithm is given in Fig. S1 in the supplemental material.

Normalization of selection pressure. Selection pressure estimates based on regression model coefficients cannot be directly compared to each other, since the degree of changes per DDD depends strongly on the baseline abundance of a selected ARG or ARG group. To facilitate a fair comparison, we normalized model coefficient (z_i) of antibiotic resistance gene *i* to the mean abundance value of *i* at the baseline (k_i). This calculation results in the normalized selection pressure (O_i), where $O_i = z_i/k_i$. The modulus of the normalized selection pressure was further log transformed to ensure appropriate visualization [$\log_{10}(|O_i|10^3)$]. The direction of the selection pressure was based on the model coefficient's sign.

Statistical analysis. D'Agostino's K-squared test was applied to check continuous variables for normality. Pearson pairwise correlation was used to determine the relationship between different metagenomic quantification methods and different similarity thresholds. A *P* value of <0.05 (two-sided) was regarded as statistically significant. All statistical analyses, including the principal component analysis (PCA), were conducted using Stata version 12.0 (Stat Corp., College Station, TX). PCA was based on eigenvalues of a correlation matrix from the ARG quantification values.

Nucleotide sequence accession number. Raw reads without human sequences were uploaded to the European Nucleotide Archive under study accession number [PRJEB10391](https://www.ebi.ac.uk/ena/record/PRJEB10391).

RESULTS

We determined intestinal resistance gene abundances and bacterial community composition in two subjects (S1 and S2) by collecting six stool samples over 34 days. During this time period, the subjects were treated with the fluoroquinolone ciprofloxacin (Cp) over a course of 6 days. The sampling of stool was performed before treatment (day 0), at days 1, 3, and 6 of treatment, and 2 and 28 days after treatment. Neither subject reported any gastrointestinal symptoms at any time.

Metagenome sequencing and database construction. Ultra-deep metagenome shotgun sequencing was applied to ensure a high sensitivity for the detection of ARGs in all samples. A total of 816,035,552 high-quality paired-end reads were generated from 12 stool samples of both subjects (55,092,349 to 89,339,172 per sample; see Table S1 in the supplemental material). Reads from all samples were assembled into 4,365,160 contigs, and 798,170 subject-specific proteins were predicted. These predicted proteins

TABLE 1 Pairwise Pearson correlation between relative abundance and ω/ω_C using the 100% sequence similarity threshold^a

ARG group	<i>r</i> for RA100/ ω 100		<i>r</i> for RA100/ ω_C 100	
	<i>P</i> value		<i>P</i> value	
Class A beta-lactamases	0.99	<0.0001	0.99	<0.0001
Class D beta-lactamases	0.99	<0.0001	0.99	<0.0001
Aminoglycoside resistance	0.98	<0.0001	0.92	<0.0001
Chloramphenicol resistance	0.99	<0.0001	0.84	<0.0001
Macrolide resistance	0.99	<0.0001	0.98	<0.0001
MEP	1.0	<0.0001	0.99	<0.0001
Glycopeptide resistance	0.99	<0.0001	0.99	<0.0001
Tetracycline resistance	0.99	<0.0001	0.99	<0.0001

^a ARG, antibiotic resistance gene; *r*, correlation coefficient; RA, relative abundance; ω , omega value; ω_C , omega CARD value; 100, 100% sequence similarity threshold; MEP, multidrug resistance efflux pumps.

were merged with proteins from the RefSeq database and the comprehensive antibiotic resistance database (CARD) (26) into a comprehensive study protein catalogue (CSPEC), which contained a total of 8,653,341 proteins, after removal of redundant and short sequences (Fig. 1).

Metagenomic antibiotic resistance gene quantification methods. Three different metagenomic ARG quantification methods have been applied to the data and were compared: (i) relative abundance (RA), (ii) omega value (ω), and (iii) omega CARD value (ω_C). Reads that mapped to annotated CARD sequences were assigned to eight ARG groups: beta-lactamase classes A and D, multidrug resistance efflux pumps (MEP), and ARGs mediating resistance to aminoglycosides, chloramphenicol, macrolides, glycopeptides, and tetracyclines. Mapped members of each group are listed in Table S2 in the supplemental material. The quantification of these ARG groups was performed by applying a 100% sequence similarity threshold, which required reads to fully concur with the ARG reference sequences at the matching region to be counted as ARG hits. Pairwise correlation between the quantification methods was executed for each ARG group by comparing RA as a reference standard with ω and ω_C (Table 1). Correlation was high between RA and ω for all ARG groups ($r \geq 0.98$, $P < 0.0001$), with only minor differences between the two methods. The same was observed for correlation between RA and ω_C , with the exception of aminoglycoside and chloramphenicol ARG groups, which exhibited a somewhat lower correlation ($r = 0.92$ and $r = 0.84$, respectively), likely reflecting the use of different reference databases. This hypothesis was supported when correlation between ω and ω_C was explored using the 100% similarity threshold (see Table S3 in the supplemental material). Once again, the chloramphenicol ARG group yielded a lower correlation coefficient of 0.85 ($P < 0.0001$).

Both ω and ω_C were further compared to determine the impact of another similarity threshold. Using 90% sequence similarity, correlation was moderate for multidrug resistance efflux pumps and the chloramphenicol ARG group ($r = 0.86$ and $r = 0.69$, respectively) and was low for the glycopeptide ARG group ($r = 0.22$), as shown in Table S3 in the supplemental material. This suggests an effect of the similarity threshold on the results of ARG abundances. However, this effect seems to depend mainly on the database used, as shown when correlating ω and ω_C with themselves at different similarity thresholds (see Table S4). Correlation generally is high when mapping against the CSPEC database (ω)

FIG 2 Comparison of real-time PCR with metagenomic quantification methodologies. *tetQ* (A and B) and *cblA* (C and D) genes were quantified using real-time PCR and metagenomic methods for both subjects. Gene abundance was normalized to a value of 1 for the highest abundance in all samples (y axis). All methods reveal similar values at each point and an equivalent progression under treatment and over time.

regardless of the similarity threshold set. This was not the case with mapping against CARD (ω_c). Applying the 100% similarity threshold generally provided the greatest concordance between all methods, most likely due to its high specificity. For this reason and because a high specificity would be crucial for clinical application of these techniques, we have performed further analysis employing the 100% similarity threshold.

Comparison of metagenomic resistance gene quantification methods with a targeted real-time PCR approach and conventional culture. To explore whether the three metagenomic ARG quantification methods are in line with an independent technique for gene abundance quantification, we performed real-time PCR for the resistance genes *tetQ* and *cblA*, both members of different ARG groups. Normalized values were highly similar for all samples in both subjects, indicating a high concordance among the quantification methods (Fig. 2). In addition, stool samples were plated on selective agar for the detection of extended-spectrum beta-lactamase-producing *Enterobacteriaceae*, vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa*. Resistant bacteria were not detected before, during, or after Cp treatment.

Effect of ciprofloxacin on the intestinal resistance gene pool. Similarities and differences in the ARG composition of the two subjects over the course of Cp administration and beyond are illustrated by principal component analysis (PCA) based on ω (Fig. 3). The first principle component (PCA 1) represents the vast majority of intersample variance (51.2%) and demonstrates primarily the response to Cp besides interpersonal differences, as can

be concluded from the different location of baseline samples (day 0). Samples are unidirectionally shifted to the left during treatment and return thereafter to the starting point (baseline sample). The vector magnitude is greater in subject 1, suggesting a more pronounced impact of Cp on the intestinal resistome than that of subject 2, whose resistome has not been altered significantly on PCA 1 from the third to the sixth day of the treatment period. However, while subject 2 was capable of an almost full ARG reconstitution 28 days after treatment, reconstitution of subject 1 was not completed. PCA 2 (19.8% variance) and PCA 3 (12.2% variance) reveal both interpersonal differences and distinct responses to Cp treatment. Differences in the ARG development for both subjects are seen mainly in the abundance profiles for the ARG group beta-lactamase class D, tetracyclines, macrolides, and chloramphenicol (Fig. 4). Of particular interest is the progression over time for class D beta-lactamases in both subjects. This group consists of four clinically important oxacillinases that had been mapped, OXA-209, OXA-347, OXA-237, and OXA-360, with the last two seen only in subject 2. While subject 1 remained at a low level throughout all sample time points, there was a strong increase in the abundance of detected OXA genes in subject 2 that was highly correlated with Cp administration and followed by a rebound to baseline 28 days after treatment (Fig. 4B). OXA-347 was found on one contig from the initial metagenomic assembly of sample TUE-S2_3 (subject 2, day 3 of treatment). On that contig, it is closely localized to an rRNA adenine N-6-methyltransferase and projects a genetic region that resembles a presumably mobile DNA element of the bacterial subclone pAC4, which was

FIG 3 Principal component analysis (PCA) of antibiotic resistance composition based on ω values. (A to C) Graphs encompass all samples of subject 1 (S1) and subject 2 (S2), with colors progressing from darker to lighter shades at later sampling points. Numbers adjacent to each sample indicate the actual sampling day before (Pre Cp), during (Cp), or after treatment with ciprofloxacin (Post Cp). (D) The variance fraction of each principal component is denoted, with the first principle component (PCA 1) comprising 51.2% of the overall variance and the first three components together comprising 83.2%.

discovered in a functional metagenomics study (27). A detailed investigation of the surrounding genetic region of OXA-347 revealed the final section of a transposase previously found in *Bacteroides fragilis* (28) located 162 bp upstream of the gene, providing additional evidence for localization on a mobile genetic element. Another interesting finding is the remainder of *acrB* as the only member of the ARG group multidrug resistance efflux pumps at day 6 of Cp treatment in subject 2 and as one of the few remaining members of this group at day 3 in subject 1. However, one hit was detected in each sample and indicates a very low abundance of *acrB*. PCA for RA and ω_C displayed a similar picture (see Fig. S2 and S3 in the supplemental material), as did resistance profiles for RA (see Fig. S4), confirming the present observations on the impact of Cp on the intestinal resistome.

The determination of the selection pressure caused by Cp is of major interest due to its frequent applications in clinical settings, for instance, for the estimation of antibiotic treatment impact on the patient's resistome in antibiotic stewardship programs. In this study, selection pressure was defined as the coefficient in an appropriate regression model (fixed effects, random effects, or ordinary least squares) and was expressed either as change in RA per defined daily dose (DDD) or as change in ω or ω_C per DDD. Table 2 shows selection pressure coefficients for all ARG groups based on ω and ω_C . A negative trend for class A beta-lactamases was

noticed (-2.66 hits per million sample reads/DDD) ($P = 0.06$), likely associated with a decline in species harboring these ARGs but not being resistant to Cp. Selection pressure coefficients for other ARG groups were less conclusive in their vectorial direction, an expected finding due to different responses of both subjects to Cp intake. Coefficient estimates based on ω were applied to normalize selection pressure (Fig. 4I). Cp treatment had its largest impact on class D beta-lactamases (positive selection) and glycopeptides (negative selection). Similar results were obtained for selection pressure coefficients when using RA quantification (see Table S5 in the supplemental material).

Effect of ciprofloxacin on the intestinal bacterial diversity and community composition. Cp administration has been found to have a fundamental impact on the gut microbiota in healthy volunteers based on 16S rRNA analysis (29, 30). Our results led us to similar conclusions. A principal coordinate analysis illustrated the distal gut microbial composition at different time points (see Fig. S5 in the supplemental material), resembling the development of the ARG intersample diversity with an almost full recovery to the initial state in subject 2 but not in subject 1.

The investigation of sample diversity after normalization to the same number of reads broadened that picture. Distal gut species diversity was profoundly disturbed over the course of Cp exposure in subject 1, as illustrated by a steep drop in diversity index values

FIG 4 Abundance profiles of antibiotic resistance gene groups (ω and ω_c). (A to H) Metagenomic quantification values ω (continuous line) and ω_c (dotted line) for various groups of antibiotic resistance genes (ARGs) are displayed in hits per million sample reads (hmr) over all sampling time points. Subject 1 (S1) is indicated by blue and subject 2 (S2) by orange. Day 0 is the baseline sample before treatment, followed by samples from days 1, 3, and 6 of Cp treatment. The final two samples were collected 2 and 28 days after treatment. (I) Comparison of the log-transformed normalized selection pressure (O_i) for all groups of ARGs reveals the strongest impact of ciprofloxacin treatment on class D BL (class D beta-lactamases) and glycopeptides. Positive values represent a positive selection, and negative values a negative selection. Values are based on ω .

representing the loss of taxa (Fig. 5). The lowest diversity occurred at the last treatment day and in the following 2 days but recovered almost fully 28 days after treatment. Subject 2 generally was much less affected, and diversity remained almost the same in all samples at values comparable to those for subject 1 under treatment, denoting a lower baseline alpha diversity.

Stream and horizon plots were generated to illustrate the phylogenetic dynamics of both subjects over all time points at the phylum level (Fig. 6). The data demonstrate the differences in the development of the intestinal microbiota for the majority of phyla in both subjects. Among the five most common phyla, both subjects responded to Cp with a similar decrease in the abundance of *Proteobacteria* but distinct from levels for the *Verrucomicrobia*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Fig. 6A). Generally, the decline in phylum abundance was more striking in subject 1, while gain and loss were fairly balanced in subject 2 (Fig. 6B), which is in accordance with the observed lower influence of Cp on bacterial gut diversity in this subject.

DISCUSSION

Antimicrobial resistance is a problem at an increasing number of hospitals across the globe (31, 32), and efforts must be intensified to prevent dissemination, which would otherwise endanger the

medical accomplishments of recent decades. The administration of antibiotics is a known risk factor for the acquisition of multidrug-resistant pathogens (10); hence, a customized prescription is an important countervailing measure. However, to do so one needs precise data about the medicinal impact of antibiotics on the human resistance gene pool. Of particular interest is the intestinal resistome, because the bacterial gut community dynamically exchanges ARGs with bacteria passing through the intestine and is easily disseminated through fecal-oral transmissions and fomites (6). In this study, we conducted a sequence-based metagenomic time series analysis to investigate the impact of ciprofloxacin on the intestinal resistome of two healthy subjects using ultradeep metagenome shotgun sequencing and a novel algorithm for the determination of the respective selection pressure.

We applied different methods for the quantification of ARGs in metagenomic data sets. Basically no differences between the methods were observed when using a highly specific 100% similarity threshold. One drawback of such a strict filter might be the inability to identify close genetic variants of ARGs. Using 90% sequence similarity reveals low correlation for several ARG groups, most strikingly when mapping was performed against CARD instead of the CSPEC database. One explanation for this is

TABLE 2 Ciprofloxacin selection pressure determination based on ω and ω_C quantification^a

ARG group	Coefficient (hmr/DDD)	95% CI	P value	Model	R ²
Mapping vs CSPC (ω)					
Class A beta-lactamases	−2.66	−5.52–0.19	0.06	FE	0.63
Class D beta-lactamases	3.49	−7.74–14.72	0.48	OLS	0.09
Aminoglycoside resistance	−0.53	−1.11–0.04	0.06	FE	0.64
Chloramphenicol resistance	−0.007	−0.27–0.014	0.15	FE	0.29
Macrolide resistance	0.33	−3.81–4.46	0.85	FE	0.64
MEP	−0.29	−4.01–3.44	0.51	FE	0.41
Glycopeptide resistance	−0.029	−0.22–0.16	0.3	FE	0.12
Tetracycline resistance	10.32	−39.97–60.61	0.23	FE	0.52
Mapping vs CARD (ω_C)					
Class A beta-lactamases	−2.89	−5.92–0.14	0.06	FE	0.62
Class D beta-lactamases	3.7	−47.16–54.56	0.53	FE	0.42
Aminoglycoside resistance	−0.58	−1.55–0.4	0.2	OLS	0.26
Chloramphenicol resistance	0.006	−0.17–0.18	0.93	OLS	0.0012
Macrolide resistance	1.08	−3.89–6.05	0.6	FE	0.8
MEP	−0.43	−5.86–5	0.5	FE	0.42
Glycopeptide resistance	−0.04	−0.28–0.2	0.28	FE	0.68
Tetracycline resistance	12.7	−58.58–84	0.27	FE	0.91

^a ARG, antibiotic resistance gene; hmr, hits per million sample reads; DDD, defined daily dose; CI, confidence interval; R², coefficient of determination; FE, fixed-effects model; OLS, ordinary least-squares regression model; CSPC, comprehensive study protein catalogue; CARD, Comprehensive Antibiotic Resistance Database; MEP, multidrug resistance efflux pumps; ω , omega value; ω_C , omega CARD value.

the usage of short reads (100 bp) in our study. A broad database like CSPC can contain proteins that have domains of high similarity to antibiotic resistance genes, and applying a best-hit algorithm would remove unspecific reads, which is not the case when mapping against a database that exclusively consists of ARGs. This phenomenon is less pronounced when using the highly specific 100% similarity threshold, as was done in this study to keep the rate of false assignments low. A high specificity is especially important when clinical decisions are based on ARG assignment from metagenomic data. Therefore, we recommend the use of a 100% similarity threshold. The performance of the studied quantification methods is very similar when employing such a highly specific filter. However, ω_C has the major advantage that the gen-

eration of a CSPC database is not required. This reduces the computational effort significantly, since a metagenomic assembly is not required unless a genomic neighborhood analysis of an ARG is desirable in individual samples. Furthermore, the addition of more samples to an analysis set would not require an adaptation of the study database. The observed progression of intestinal ARGs over the course of treatment was confirmed by a targeted PCR approach. This indicates high similarity between these techniques and supports that metagenomic quantification methods are very precise. Although metagenomic approaches are still considered costly and time-consuming compared to standard targeted PCRs, this view is challenged when the human resistome is the object of investigation. A quantitative PCR panel would need to include hundreds of ARG targets per sample to cover the same spectrum as our metagenomic approach; thus, it is not likely to be less time-consuming or expensive. A metagenomic approach also provides the possibility to immediately reanalyze data when new ARGs become known without the need for additional data collection.

The administration of Cp had different effects on the two subjects in a number of ARG groups, namely, the class D beta-lactamases. One reason might be that these oxacillinases were present in species that possessed determinants of ciprofloxacin resistance in one subject but not in the other. Cp resistance in species usually would lead to an increase in the population size during Cp treatment regardless of the concurrent presence of ARGs that mediate resistance to other substances. However, these genes would increase in frequency, too. A similar effect has been observed in a study where the administration of nonfluorquinolone antimicrobials was associated with an increased abundance of *qnrB* that confers resistance to fluorquinolones (33). To what extent this mechanism played a role in the present study is difficult to assess, but the difference in the baseline status of the intestinal microbiota in both subjects indicates that species composition varied to a

FIG 5 Alpha species diversity statistics. Two measures of alpha diversity estimation (Shannon and Simpson indexes) were applied for subject 1 (S1) and subject 2 (S2) after rarefying to the same number of reads for all samples. Diversity index values are shown on the y axis, while the x axis reflects sampling time points.

FIG 6 Microbiota community evolution over the course of ciprofloxacin treatment. Computations were performed after normalization to an equal number of reads for all samples. (A) Stream plots show the phylogenetic dynamics of the five most abundant phyla in subjects 1 and 2. The width of the stream is directly proportional to the phylum abundance. Values are smoothed using a square root transformation of the normalized read counts. Stream plots were generated using the streamgraph *htmlwidge* R package. (B) Horizon plots are displayed for 26 phyla over the time of ciprofloxacin treatment and afterwards. Color changes to red indicate a decreasing abundance compared with the baseline sample (day 0), and changes to blue indicate increasing abundance. Color intensity corresponds to the degree of change. Dark colors reflect a sharp change and light colors a mild change, as also indicated by the bar on the right that shows the magnitude of changes (Δ values; see Materials and Methods for details). Horizon plots were generated using the *lattice extra* R package.

significant degree and that both subjects indeed had a distinct initial situation.

There is an alternative explanation for the strong positive selection of oxacillinases. ARGs located on mobile genetic elements can be released from dying bacteria and are incorporated by Cp-resistant survivors, which subsequently would amplify even non-Cp-related ARGs while gaining dominance in the community. Because such mobile ARGs can contribute to the common pool of resistance genes by circulating among bacteria, potentially from commensals to pathogenic species, they should be ranked higher according to their associ-

ated public health risk, as recently suggested (34). Genetic mobility also plays a role in the evolution of antimicrobial resistance, since a gene might not confer resistance to its native host but when expressed in other hosts (35), mirroring the complex relationship between resistance genotype and phenotype. Thus, the exploration of the genetic mobility of an ARG is another crucial step for ranking ARGs according to their public health hazard. However, a gene's risk of dissemination cannot be derived from its simple presence in a metagenomic data set (36) but from its genetic and genomic context through an analysis of assembled metagenomic data.

We have found OXA-347 likely to be located on a mobile genetic element because of a transposase element located upstream on the respective metagenomic contig. The lagging increase of oxacillinases after 3 days of treatment in subject 2 is further indicative of a horizontal gene transfer. Even though reads from the other oxacillinases could not be assembled into contigs due to their lower abundance in the sample data set, it is possible that they also reside on mobile genetic elements.

Apart from genetic mobility, oxacillinases generally are considered important resistance factors. For instance, OXA-237 has a proven carbapenem-hydrolyzing activity (37), a mechanism of high clinical relevance due to the loss of an important class of bactericidal antibiotics that often are used for the treatment of severe infections. The observed rise in oxacillinase gene abundance during the course of treatment poses a serious threat for patients, as any infection originating from the gut may be more difficult to treat. The risk of resistance dissemination additionally would increase and presents a hazard for close contacts. These alarming findings originated from sequenced-based metagenomics while cultural screening for resistant bacteria remained negative, an indication for the potential superiority of the sequencing approach for studies of resistance, perhaps even for diagnostic purposes, since active screening of cultures as performed in our study reflects a typical routine screening.

As mentioned earlier, some ARGs pose a higher public health threat than other members of the same ARG group. For the investigation of clinically and hygienically relevant treatment effects, it appears more important to focus on those single high-risk ARGs. We have outlined the impact on ARG groups rather than single genes due to a possible unspecific assignment of short reads to members of the same ARG group. The introduction of sequencing techniques that generate longer read lengths at lower prices has the potential to overcome this limitation, given a suitable sequencing depth.

Selection pressure was determined for all ARG groups. A trend to a negative selection was observed for class A beta-lactamases ($P = 0.06$). Selection pressure estimates of other ARG groups lacked statistical significance. This is explained mainly by the different response of both subjects to CP administration. Generally, the highest normalized effect of Cp administration was observed in the ARG groups class D beta-lactamases and glycopeptides, and the lowest effect was in macrolide ARGs (Fig. 4I). Both positive and negative selection has been noted. While these results demonstrate that the effect of an antibiotic is not necessarily unidirectional for all ARGs and ARG groups, they also must be interpreted with caution. The estimation is based on model coefficients of only two subjects and needs to be confirmed on larger cohorts or in clinical studies with an appropriate sample size.

The presence of genes that confer resistance to ciprofloxacin is of particular interest due to the respective treatment administered to the participants. Resistance to fluoroquinolones can be mediated by Qnr proteins that protect type II DNA topoisomerases from inhibition (38, 39). *qnr* genes usually are located on plasmids and have been reported worldwide with a prevalence of up to 54% (27, 40), but they have not been found in the two subjects. Efflux systems can cause fluoroquinolone resistance as well. Genes associated with efflux systems usually belong to the core genome of bacterial species and can have various functions; therefore, they are not classical ARGs (41). Resistance caused by efflux systems might occur or is amplified through mutations that induce a change in the expression of pump components (42). Thus, their

finding in a sample must be interpreted with caution. Nevertheless, we have detected *acrB* under Cp administration, while other members of the multidrug resistance efflux pumps were not traceable anymore. *acrB* is the pump that is associated with the AcrAB-TolC tripartite multidrug-resistant efflux pump system that has been reported to confer resistance to ciprofloxacin, especially in the presence of alterations in *acrB* (43). Some authors regard *acrB* as a *de facto* ARG due to its high expression in pathogenic isolates like *Pseudomonas aeruginosa* and *Escherichia coli* (35). Although this is a strong lead that efflux systems conferring ciprofloxacin resistance were present in the samples and selected under treatment, the low number of *acrB* hits underlines a minor effect. Other mechanisms of Cp resistance might have been more prominent, perhaps resistance facilitated by amino acid alteration in GyrA, GyrB, ParC, and ParE, which typically are located in the amino terminus that contains the enzymes' active site (42). However, the investigation of enzyme alterations is intricate. As long as sequence reads do not span the whole target gene length, it is not possible to reliably estimate the frequency of such resistance mechanisms.

We have introduced a novel method to determine the actual selection pressure of an antibiotic agent based on metagenomic ARG quantification and regression models that take a panel of data from individuals into account and can correct for potential confounders. The method is intended for implementation in clinical studies that aim to estimate the impact of an antibiotic treatment on single ARGs or ARG groups in patients. For the determination of a general antibiotic effect on a patient population, for instance, in observational studies or as the readout for randomized controlled trials, it is crucial to investigate a sufficient number of patients and to monitor various time points during treatment as well as prior to treatment to account for intraindividual variability over time. Differences in how an antibiotic affects the intestinal resistome can be unveiled, providing valuable information for clinicians and those responsible for infection control, since administrations of antibiotics can be adapted to agents with the lowest selection pressure when appropriate in the clinical situation. Future improvement of sequencing techniques and their availability to laboratories with fewer resources will further promote the utilization of this novel analysis method.

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