Antibiotic Resistance Gene Detection in the Microbiome Context

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Within the past decade, microbiologists have moved from detecting single antibiotic resistance genes (ARGs) to detecting all known resistance genes within a sample due to advances in next generation sequencing. This has provided a wealth of data on the variation and relative abundances of ARGs present in a total bacterial population. However, to use these data in terms of therapy or risk to patients, they must be analyzed in the context of the background microbiome. Using a quantitative PCR ARG chip and 16S rRNA amplicon sequencing, we have sought to identify the ARGs and bacteria present in a fecal sample of a healthy adult using genomic tools. Of the 42 ARGs detected, 12 fitted into the ResCon1 category of ARGs: *cfxA*, *cphA*, *bacA*, *sul3*, *aadE*, *bla*$_{TEM}$, *aphA1*, *aphA3*, *aph(2′)-Id*, *aacA/aphd*, *catA1*, and *vanC*. Therefore, we describe these 12 genes as the core resistome of this person's fecal microbiome and the remaining 30 ARGs as descriptors of the microbial population within the fecal microbiome. The dominant phyla and genera agree with those previously detected in the greatest abundances in fecal samples of healthy humans. The majority of the ARGs detected were associated with the presence of specific bacterial taxa, which were confirmed using microbiome analysis. We acknowledge the limitations of the data in the context of the limited sample set. However, the principle of combining qPCR and microbiome analysis was shown to be helpful to identify the association of the ARGs with specific taxa.

Keywords: microbiome, resistome, feces, human

Introduction

To minimize the burden of antibiotic resistance and to identify the areas of greatest risk to human health, we must understand how antibiotic resistance genes (ARGs) selection and proliferation occur in a complex bacterial system such as the human gut microbiome. There is not yet a consensus on the selection of ARGs and bacterial changes required for the proliferation of ARGs and increases in ARG abundances in complex bacterial populations.[1–8] To understand the influence of antibiotics on mixed complex populations, such as the gut microbiome, we must first identify and understand the background or baseline resistance genes and intrinsic resistance mechanisms present in the human gut bacteria. Only then can we identify the risks and potential pathways of ARG transfer from the gut microflora to pathogenic bacteria. If an ARG identified in the gut microbiome is present on the chromosome of an anaerobe, it does not pose the same risk to the treatment of a patient as if the same gene is present on a highly mobile plasmid. Thus, the genes must be identified in their bacterial or microbiome context.

The aims of this study were to identify and measure the relative abundances of ARGs and bacteria present in a fecal sample of a healthy adult using molecular biology tools. As we move further toward genomic analysis of ARGs and bacteria, we must generate guidelines for the interpretation of the data generated. To do this, we must understand which bacteria and ARGs are present in the healthy human and then what constitutes a risk to the treatment of a patient in terms of likelihood of transfer to pathogenic bacteria.

Materials and Methods

Sample preparation and DNA extraction

A fecal sample was collected from a healthy adult who had taken no antibiotics in the previous 2 years. It was immediately homogenized and 0.6 g was added directly to the Mo-Bio™ Power Soil® DNA isolation kit tube in step one of the protocol. The DNA was extracted using the kit protocol.

The NUI Maynooth Biomedical and Life Sciences Research Ethics Sub-Committee approved this study and

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experimental protocols (application reference number BRSERC-2014-007). All methods were performed in accordance with ethical guidelines and regulations. Written informed consent was obtained from the subject.

16S rRNA amplicon sequencing

The V3 and V4 regions of the 16S rRNA genes were amplified and sequenced using the Illumina MiSeq® primers and protocol.[9] A 2×150 paired-end configuration was used for sequencing. The image analysis and base calling were processed using MiSeq Control Software. The data were quality control checked and trimmed and analyzed initially using the Illumina BaseSpace app.[10] The sequencing data were processed using Quantitative Insights Into Microbial Ecology version 1.5.0.[11] Shannon diversity, collectors curve, and Chao-1 were used to determine the evenness of the 16S rRNA results.[12]

ARG relative abundance (qPCR)

DNA extracted from the fecal samples was used to analyze the relative abundances of the ARGs using a highly parallel quantitative PCR (qPCR) platform (Wafergen Smartchip).[13] The samples analyzed comprised three biological replicates for each sample and three technical replicates of each biological replicate. The samples were analyzed for the relative abundance of 384 primer sets targeting known ARGs and mobile genetic elements (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/mdr). The C_t values of each primer pair were normalized using the 16S rRNA gene values (delta$C_t = C_t$ARG–C_t16SrRNA). Results with a C_t value of >28 were removed. The deltaC_t values and fold change were calculated according to Relative Gene Expression Data Using Real-Time Quantitative PCR and the deltadeltaC_t method.[14] The SmartChip has been validated by comparison with metagenomics.[15]

Results

ARG resistome

Forty-two different ARGs (plus six mobile elements and two repressor genes) were detected in the fecal sample (Table 1). Using the definitions of ARGs provided by Martinez *et al.* would exclude efflux genes, *ampC* genes, the erythromycin resistance genes, and the tetracycline resistance genes as these are not considered ARGs from an ecological viewpoint.[16] The erythromycin and tetracycline resistance genes are frequently identified on the chromosomes of anaerobes commonly found in the human gut microbiome and are not considered to confer resistance in these bacteria. Their presence identified the bacteria harboring these genes, rather than a resistance reservoir. Of the 42 ARGs detected, 12 fitted into the ResCon1 category of ARGs as defined by Martinez *et al.*[16] These were ARGs *cfxA, cphA, bacA, sul3, aadE, aphA1, aphA3, aph(2′)-Id, aacA/ aphd, bla*TEM*, catA1,* and *vanC.* Therefore, we describe these 12 genes as the core resistome of this person's fecal microbiome and the remaining 30 ARGs as descriptors of the microbial population within the fecal microbiome.

Relative abundances of genes

The relative abundances of each ARG were determined by comparison with the 16S rRNA gene abundance. This en-

sured that variations in DNA quantities were not responsible for variations in the ARG abundances in the sample (Table 1). The genes with the highest relative abundances comprised mobile genetic elements (insertion sequences and transposases), tetracycline and erythromycin resistance genes associated with the anaerobes present in the human gut microbiome, and the *cfxA* beta-lactamase gene. The genes detected in the lowest relative abundances comprised efflux genes (*tetC, ceoA,* and *tetB*), a tetracycline resistance gene regulator (*tetR*), *vanC,* mobile genetic elements (*IncN* and *pNI105*), beta-lactamase (*bla*TEM), aminoglycoside (*aacA/ aphd*), and chloramphenicol (*catA1*) resistance genes.

Bacterial community analysis using 16S rRNA gene amplicon sequencing

Microbial composition. The phyla, which constituted the taxa at >1% within the 89303 OTU sequence reads, that passed the quality control filtering comprised Firmicutes (66%), Bacteroidetes (28%), Proteobacteria (2.5%), unclassified (1.5%), and Actinobacteria (1.2%). The dominant phyla are consistent with previous findings.[16] Within these phyla, the taxa were spread across 32 classes, with 6 of those representing >1% relative abundances: Clostridia (65%), Bacteroidia (20%), Flavobacteria (7.3%), unclassified (2%), Actinobacteria (1.2%), and Bacilli (1%). The classes were subdivided into 68 orders with Clostridiales (64%), Bacteroidales (20%), Flavobactriales (7.3%), and unclassified (2.3%), representing those at >1%. The families within the microbiome (*n* = 143) at >1% were represented by Lachnospiraceae (32%), Ruminococcaceae (25%), Bacteroidaceae (11%), Flavobacteriaceae (7.3%), unclassified (4.6%), Clostridiaceae (3.8%), Paraprevotellaceae (3.7%), Odoribacteraceae (2.7%), Porphyromonadaceae (2.2%), and Eubacteriaceae (1.6%). A total of 263 different genera were represented in the fecal microbiome and those with >1% relative abundance comprised 19 different genera (including unclassified), which represented 90% of the total microbiome composition (Table 2). Dominant genera also agree with those previously detected in the greatest abundances in fecal samples of a healthy human.[17] Although correlations between bacterial phylogenies and antibiotic resistomes have been reported by Pehrsson *et al.*, this was using diverse habitats.[18]

Discussion

In a mixed bacterial population, such as human feces, bacteria are present that contain ARGs either on their chromosomes or on mobile elements. These bacteria are maintained within the fecal population due to their roles, regardless of the ARG or selective antibiotic pressures. With advances in next generation sequencing (NGS) came studies measuring the influences of antibiotics on complex bacterial populations and their total antibiotic resistomes, such as the human gut microbiome.[1–8] However, to identify the changes occurring within a population of bacteria, we must first identify the ARGs present in the natural fecal bacterial population, independent of selective pressure. With reduced cost and increased capacity, NGS has become a potential tool for the identification of ARGs and pathogens directly from patients. In order for such technology to function, one must understand the difference between carriage and selection of bacterial species and ARGs. This study aimed to

TABLE 1. RELATIVE ABUNDANCES OF ANTIBIOTIC RESISTANCE GENES DETECTED USING THE QUANTITATIVE PCR CHIP

Primer name	Role	Antibiotic class	Average Ct value	Standard deviation of Ct values	Relative abundances ranked high to low	Example of fecal bacteria harboring gene on chromosome	Family present in sample
16S rRNA	16S rRNA		12.53	0.110			
IS613	MGE	Insertion sequence	15.90	0.109	0.096722812		
tetQ	Protection	Tetracycline	16.71	0.155	0.055168937	Bacteroides fragilis	Bacteroidaceae
tetW	Protection	Tetracycline	16.81	0.146	0.051474439	Bifidobacterium longum	Bifidobacteriaceae
tetO	Protection	Tetracycline	16.92	0.149	0.0476956	Clostridium colicanis	Clostridiaceae
Tp614	MGE	Transposase	17.17	0.222	0.040107059		
cfxA	Deactivate	Beta lactam	17.75	0.127	0.02683017		
ermB	Protection	MLSB	18.41	0.143	0.016980232	Clostridium perfringens	Clostridiaceae
ermF	Protection	MLSB	18.89	0.211	0.012174447	Eubacterium spp.	Eubacteriaceae
tetx	Deactivate	Tetracycline	20.32	0.087	0.004518313	Flavobacterium spp.	Flavobacteriaceae
mefA	Efflux	MLSB	20.48	0.174	0.004044004		
tet(32)	Protection	Tetracycline	20.54	0.155	0.003879268	Unknown	
ISEfm1-Entero	MGE	Insertion sequence	22.00	0.102	0.001410087		
tnpA	MGE	Transposase	22.19	0.157	0.00123609		
sulIII-marko	Protection	Sulfonamide	22.91	0.025	0.000750427		
matA/mel	Efflux	MLSB	23.23	0.066	0.000601145		
aphA3	Deactivate	Aminoglycoside	23.40	0.179	0.000534323		
tetM	Protection	Tetracycline	23.43	0.064	0.000523327	Streptococcus mutans	Streptococcaceae
sat4	Efflux	MDR	23.47	0.095	0.000509016		
aph(2′)-Id	Deactivate	Aminoglycoside	23.94	0.033	0.000367492		
ermT	Protection	MLSB	24.60	0.073	0.000232578	Streptococcus pyogenes	Streptococcaceae
msrC	Efflux	MLSB	24.67	0.237	0.000221562		
ampC	Deactivate	Beta lactam	24.71	0.44	0.000215504	Pseudomonas spp.	Pseudomonadaceae
mdtF	Efflux	MDR	24.81	0.376	0.000202471		
tetL	Efflux	Tetracycline	24.84	0.384	0.000196934		
yidY/mdtL	Efflux	Amphenicol	24.90	0.251	0.000188912		
bacA	Deactivate	other	25.03	0.127	0.000172633	Escherichia coli	Enterobacteriaceae
aadE	Deactivate	Aminoglycoside	25.46	0.449	0.000128139	Streptococcus anginosus	Streptococcaceae
acrF	Efflux	MDR	25.52	0.242	0.000122919		
yceL/mdtH	Efflux	MDR	25.55	0.320	0.00012039		
acrR	Regulator	MDR	25.57	0.283	0.000118732		
yceE/mdtG	Efflux	MDR	25.61	0.362	0.000115486		
acrB	Efflux	MDR	25.72	0.502	0.000107008		
aphA1	Deactivate	Aminoglycoside	25.75	0.312	0.000104805		
mdtE/yhiU	Efflux	MDR	25.83	0.336	9.91519E-05		
tetPB	Protection	Tetracycline	25.92	0.129	9.31555E-05	C. perfringens	Clostridiaceae
acrA	Efflux	MDR	26.02	0.442	8.69171E-05		
cphA	Deactivate	Beta lactam	26.06	0.178	8.45404E-05		
tetPA	Efflux	Tetracycline	26.14	0.412	7.99801E-05		
bexA	Efflux	MDR	26.27	0.207	7.30883E-05		
tolC	Efflux	MDR	26.82	0.158	4.99208E-05		
tetB	Efflux	Tetracycline	26.82	0.179	4.99208E-05		
catA1	Deactivate	Amphenicol	26.87	0.201	4.82203E-05		
pNI105map-F	MGE	plasmid replication	27.19	0.136	3.86278E-05		
ceoA	Efflux	Amphenicol	27.19	0.195	3.86278E-05		
tetR	Regulator	Tetracycline	27.22	0.069	3.78329E-05		
bla_{TEM}	Deactivate	Beta lactam	27.25	0.521	3.70543E-05		
aacA/aphD	Deactivate	Aminoglycoside	27.32	0.206	3.52993E-05		
IncN_rep-1_f	MGE	Plasmid incompatibility	27.33	0.422	3.50555E-05		
vanC	Protection	Vancomycin	27.34	0.386	3.48133E-05		
tetC	Efflux	Tetracycline	27.42	0.323	3.29354E-05		

Three technical replicates were averaged to measure the Ct values for each primer set.
MDR, multidrug resistance; MGE, mobile genetic element.

TABLE 2. MICROBIOME PROFILES OF THE HUMAN
FECAL SAMPLE (>1%) AT GENERA LEVEL

Genus	% Relative abundance
Faecalibacterium	20.46739751
Bacteroides	11.02874483
Blautia	8.159860251
Roseburia	8.006449951
Unclassified	7.093826635
Dorea	4.151036359
Ruminococcus	3.951714948
Flavobacterium	3.84085641
Paraprevotella	3.721039607
Pseudobutyrivibrio	3.398542042
Polaribacter	3.33135505
Coprococcus	2.032406526
Oscillospira	1.923787555
Parabacteroides	1.675195682
Acetobacterium	1.577774543
Clostridium	1.537462347
Butyricimonas	1.436681858
Odoribacter	1.294469391
Lachnospira	1.11642386

integrate NGS and antibiotic resistance qPCR chip technology to describe the fecal population of bacteria and the relative abundance of their resistome in a healthy human under no antibiotic selective pressure.

The published data on the human gut microbiome alterations due to antibiotics are highly variable. To date, there have been no definite conclusions on the bacterial genera that proliferate or decrease after specific antibiotic administration. There is no consensus on whether any microbiome changes occur at all or whether intersubject variability was greater than the effects of the antibiotics administered. The first studies of the human gut microbiome tried to identify and describe the core set of bacterial taxa responsible for health and disease. However, such studies among healthy individuals revealed wide variation in the taxonomic composition of the microbiome, which prevented the discovery or identification of a core microbiome.[19,20]

The majority of the ARGs detected may have been associated with the presence of specific bacterial taxa. There was a high proportion of mobile genetic elements detected, which suggests high genetic mobility within the fecal microbiome and the detection of several ARGs, which are not associated with intrinsic resistance of common gut microbiome bacteria. The conclusion from this study is that although many ARGs can be detected and their abundances measured using DNA-based tools, we must put these genes in the context of the bacterial composition of the sample, in this case feces from a healthy human, to identify the genes, which pose a risk to the treatment of pathogenic infections. This study highlights the need to put DNA analysis in context and has listed several ARGs that may be present on the chromosome of the natural fecal microbiome. The conclusions may not be further extrapolated due to the small sample size.

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