IDENTIFICATION OF AMINOGLYCOSIDE PHOSPHOTRANSFERASES OF CLINICAL BACTERIAL ISOLATES IN THE MICROBIOTA OF RUSSIANS

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Antibiotic resistance is one of the biggest threats to modern medicine. Response to antimicrobial treatment is seriously disrupted by aminoglycoside phosphotransferases (Aph) — enzymes produced by bacteria. The *aph* genes were annotated in many bacterial species, including commensals of the gut microbiota that can transfer these genes to clinically important strains. For this study we prepared a catalog of 21 *aph* genes. The *in silico* analysis of 11 intestinal microbiomes of healthy Russians revealed the presence of 3 cataloged *aph* genes in 7 microbiota samples, namely *aph(3'')-Ib*, *aph(3')-IIIa* and *aph(2'')-Ia*. The most frequent was the *aph(3')-IIIa* gene detected in 6 metagenomes. Of note, this gene was first discovered in Enterococcus faecalis, but in this study we observed it in sequences typical for commensal *Ruminococcus obeum* and opportunistic *Enterococcus faecium*, *Roseburia hominis*, *Streptococcus pyogenes* and *Staphylococcus epidermidis*. Similarly, *aph(2'')-Ia* originally present in *E. faecalis* was detected in a sequence typical for *Clostridium difficile*. Our findings are consistent with the reports on the strong association between the geographical origin of the individual and frequency of *aph* genes. We suggest that clinical examination should include antibiotic sensitivity tests run not only on the causative agent, but also on the gut microbiota, for a better treatment outcome.

Keywords: antibiotic resistance, aminoglycoside phosphotransferase (Aph), clinical isolates of bacteria, human gut microbiota

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ИДЕНТИФИКАЦИЯ АМИНОГЛИКОЗИДФОСФОТРАНСФЕРАЗ КЛИНИЧЕСКИХ ШТАММОВ БАКТЕРИЙ В МИКРОБИОТЕ ЖИТЕЛЕЙ РОССИИ

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Устойчивость бактерий к антибиотикам является одной из самых серьезных проблем в современной медицине. Эффективность антимикробной терапии снижается вследствие работы бактериальных ферментов — аминогликозидфосфотрансфераз (Aph). Гены *aph* аннотированы в геномах многих бактерий, в том числе комменсалов микробиоты кишечника, из геномов которых они могут попадать в геномы клинически значимых штаммов. Анализ *in silico* 11 метагеномов кишечника здоровых людей из России показал наличие в 7 образцах микробиоты 3 генов *aph* из 21, включенного в каталог, составленный для исследования: *aph(3'')-Ib*, *aph(3')-IIIa* и *aph(2'')-Ia*. Наиболее распространенным оказался ген *aph(3')-IIIa*, найденный в 6 исследованных метагеномах. Важно, что этот ген впервые был обнаружен у *Enterococcus faecalis*, но в данной работе он был идентифицирован в генетическом окружении, характерном для комменсальной бактерии *Ruminococcus obeum* и условно-патогенных бактерий *Enterococcus faecium*, *Roseburia hominis*, *Streptococcus pyogenes* и *Staphylococcus epidermidis*. То же наблюдали для гена *aph(2'')-Ia*: он был обнаружен для *Clostridium difficile*, а не для *E. faecalis*. Полученные результаты согласуются с литературными данными, указывающими на значимое влияние географического происхождения людей на распространенность *aph*-генов. Также, учитывая данные исследования, представляется обоснованным при клиническом обследовании пациентов с инфекционными заболеваниями и назначении антибиотиков для их лечения анализировать антибиотикорезистентность не только бактерии-возбудителя, но и микробиоты пациента.

Ключевые слова: устойчивость к антибиотикам, антибиотикорезистентность, аминогликозидфосфотрансферазы, Aph, клинические штаммы бактерий, микробиом, микробиота, кишечник человека

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At least 2 million people in the USA become infected with antibiotic-resistant bacteria every year, and at least 23,000 people die of these bacterial infections [1]. The growing antibiotic resistance of human pathogens is a serious threat to global health and has a significant impact on the environment. According to Antibiotic Resistance Genes Database (ARDB) [2], 13,293 antibiotic resistance genes of microorganisms have been discovered so far. Transfer of genetic elements between bacteria through intricate routes in mixed microbial communities promotes dissemination of resistance genes [3].

The human gut is home to about 10^{14} microbial cells and approximately 1000 microorganisms [4]. It is a dynamic reservoir of antibiotic resistance genes termed the resistome [5]. Antibacterial treatment has a significant impact on the gut resistome: it stimulates horizontal gene transfer and exerts selective pressure on its members [6]. Studies of gut microbiota residents resistant to antibiotics show that commensals of the human gut can also be a source of resistance genes for other bacteria, including pathogenic strains [7].

Studies of antibiotic resistance employ various cutting-edge technologies and methods, such as next generation sequencing, bioinformatic analysis, or analytical chemistry, making it possible to identify up to 30 gene clusters associated with antibiotic resistance [8]. Researchers of the Center for Genome Sciences and Systems Biology, Washington University School of Medicine, analyzed genes responsible for resistance to 18 clinically relevant antibiotics across ecologies. The bioinformatic analysis identified genes conferring resistance to two antibiotics widely used in the clinical setting and agriculture: β-lactams and tetracyclines [9].

Antimicrobial therapies can be seriously disrupted by aminoglycoside phosphotransferases (Aph) [10]. Genes that encode these enzymes were first discovered in plasmids and mobile elements of clinical strains of gram-positive and gram-negative bacteria [11]. As demonstrated by the phylogenetic analysis of Aph of clinical strains and strains producing aminoglycoside antibiotics [12], aminoglycoside phosphotransferases can be organized in 7 groups depending on the enzyme-modified position of the hydroxyl group of the antibiotic: Aph(2''), Aph(3'), Aph(3''), Aph(4), Aph(6), Aph(7'') and Aph(9).

Aph-encoding genes have been annotated in many bacterial genomes, including non-pathogenic strains of the gut microbiota from where they can transfer to clinical strains [13]. Metagenomic DNA isolated from the human neonatal gut was shown to carry multiple genes conferring resistance to aminoglycosides and β-lactams [14].

A comparative study of 832 human gut metagenomes obtained from the residents of 10 different countries (England, Finland, France, Italy, Norway, Scotland, USA, Japan, China, and Malawi) established that the diversity of resistance genes was largely dependent on the geographical origin of the participant [15].

The spread of aph genes was studied in many laboratories worldwide. The *aac(6')-Ie-aph(2'')-Ia* gene was found to be the most prevalent gene of enterococcal aminoglycoside resistance; it was detected in 26 out of 27 isolates obtained from patients of an Iranian hospital [16]. The epidemiologic study of 543 clinical strains isolated from Japanese patients showed that of 12 studied genes of aminoglycoside-modifying enzymes, one — the *aph(2")-Ie* gene- was isolated from 3 strains of *Enterococcus faecium* and another one –*ant(9)-Ia* — was detected in *E. faecalis*, *E. faecium* and *E. avium*. Nucleotide sequences of *ant(9)-Ia* in these 3 enterococci were identical to those of *Staphylococcus aureus* and were harbored on transposon Tn554 [17]. Because aminoglycosides are often used to treat staphylococcal infections, a study was carried out to estimate the prevalence of aminoglycoside resistance among methicillin-resistant strains of *S. aureus* isolated from patients of an Iranian hospital. Genes *aac(6')-Ie-aph(2")*, *aph(3')-IIIa* and *ant(4')-Ia* were detected in 134 (77.0 %), 119 (68.4 %) and 122 (70.1 %) isolates, respectively [18].

In light of the above, identification of aminoglycoside phosphotransferases in the gastrointestinal metagenomes of Russian residents becomes a pressing issue.

METHODS

Sample preparation and DNA sequencing

We studied the gut microbiota of 11 healthy individuals of different sex and age, all residents of Moscow and Tver, Russia. Stool samples were collected using standard techniques [19]. Samples were frozen at –80 °C until further analysis.

DNA was extracted from weighted amounts of frozen stools using the QIAamp Fast DNA Stool Mini kit (Qiagen, Germany) according to the vendor's protocol with optimized lysis conditions for microbial DNA extraction (Isolation of DNA from Stool for Pathogen Detection, Qiagen, USA). The concentration of the obtained DNA was measured using the Qubit Fluorometer (Invitrogen, USA). The obtained genomic DNA was fragmented using the Covaris M220 focused ultrasonicator (Covaris, USA) to achieve fragment length between 100 and 700 b. p. (average size was ~350 b. p.).

Libraries for further sequencing were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina (NEB, UK). Fragments ranging from 250 to 500 b. p. (adapter sequences included) were selected for further sequencing. Quality control of the obtained libraries was performed on the Agilent TapeStation (Agilent Technologies, Germany); the libraries were mixed in equimolar amounts. Adapter sequences used at library prep step were as follows: Read1 (AGATCGGAAGAGCACACGTCTGAA CTCCAGTCACNNNNNNATCT CGTATGCCGTCTTCTGCTTG) and Read2 (AGATCGGAAGAGCGTCGTGTAGGGAAAGAG TGTAGATCTCG GTGGTCGCCGTATCAT), where NNNNNN is a 6-nucleotide index unique for each sample. After quality control was performed and library molecules were counted by quantitative PCR, the libraries were sequenced on one lane of Illumina HiSeq 4000 (101 cycles per each fragment's end) using the HiSeq 4000 SBS sequencing kit ver. 1 (Illumina, USA). FASTQ files were obtained using bcl2fastq v2.17.1.14 Conversion Software (Illumina). Quality scores were encoded as Phred 33. The obtained metagenomes were uploaded to the Sequence Read Archive (SRA) NCBI. They are presented in Table 1.

Quality control of metagenomic libraries and read assembly

Quality control of the resulting metagenomic libraries was performed using FastQC [20]. Read trimming was done using trimmomatic [21]. Contaminating host DNA was filtered by aligning the metagenomic reads against the human genome. Alignment was performed using Bowtie2 [22]. The metagenomic reads were assembled into contigs using SPAdes [23]. Description of the assembled reads is provided in Table 2.

Compiling a catalog of aminoglycoside phosphotransferases-encoding genes

Drawing upon the literature [12], we compiled a catalog of aminoglycoside phosphotransferase-encoding genes

isolated from the clinical strains of *Acinetobacter baumannii*, *Alcaligenes faecalis*, *Bacillus circulans*, *Burkholderia pseudomallei*, *Campylobacter jejuni*, *Enterococcus faecalis*, *Escherichia coli*, *Enterococcus casseliflavus*, *Enterococcus faecium*, *Legionella pneumophila*, and *Pseudomonas aeruginosa*. The catalog listed 21 gene. We also compiled a catalog of amino acid residues encoded by the selected Aph genes.

Metagenomic analysis

A Perl script was written to run the BLASTX search for aminoglycoside phosphotransferase genes in the assembled contigs and to filter the results by 2 parameters: homology and relative alignment length. The search was performed in the catalog of 31 amino acid sequences prepared in advance. Sequence alignments generated by BLASTIX were filtered by homology and relative alignment length. Relative alignment length was calculated as

$$L_{relative} = \frac{L_{alignment}}{L_{sequence}},$$

where $L_{alignment}$ is the length of the obtained alignment and $L_{sequence}$ is the length of the reference amino acid sequence from the catalog. We did not intend to screen the samples for new aminoglycoside phosphotransferase genes, therefore for homology the minimal value was set to 90 %, and the minimal alignment length was set to 80 %. To profile the species present in the studied samples, MetaPhlAn2 was used [24].

RESULTS

Compiling a catalog of aminoglycoside phosphotransferase genes of clinically relevant strains

Depending on the position of the enzyme-modified hydroxyl group of the antibiotic, aminoglycoside phosphotransferases were distributed into 7 subgroups: Aph(2''), Aph(3'), Aph(3''), Aph(4), Aph(6), Aph(7''), and Aph(9). The catalog of genes of clinical strains was prepared by summing up the data from the review [12]. The catalog of aminoglycoside phosphotransferase-encoding genes of clinically relevant bacterial strains is provided in Table 3.

Screening Russian metagenomes for aminoglycoside phosphotransferase genes

Using the Perl script, we analyzed gut metagenomes of 11 healthy Russian individuals. The results are presented in Table 4. It total, we identified 3 *aph* genes in 7 metagenomes. All genes were identified with 100 % homology. Of these 3 genes, the most prevalent was gene aph(3')-IIIa: it was missing in only one metagenome (D5F). Two *aph* genes, namely *aph(2'')-IIa* and *aph(3')-IIIa*, were present only in metagenome D12F. Gene *aph(3'')-Ib* was detected in only one metagenome (D5F).

The studied metagenomes were profiled for species diversity using MetaPhlAn2. Reads unambiguously assigned to bacterial species were aligned against metagenomic contigs using Bowtie2. Thus, contigs that carried aminoglycoside

Table 1. The studied metagenomes

№	Sample	Sex	Age, years	Region	Genbank ID
1	4B_S2	F	34	Tver, Russia	SRX1870055
2	12_S1	F	28	Tver, Russia	SRX1878777
3	D3F	M	15	Moscow, Russia	SRX2672491
4	D4F	M	15	Moscow, Russia	SRX2672492
5	D5F	M	15	Moscow, Russia	SRX2672493
6	D6F	M	15	Moscow, Russia	SRX2672494
7	D11F	M	15	Moscow, Russia	SRX2672495
8	D12F	F	15	Moscow, Russia	SRX2672496
9	D13F	F	15	Moscow, Russia	SRX2672497
10	DG_S1	F	28	Tver, Russia	SRX1869842
11	HG550	F	6	Tver, Russia	SRX1869839

Table 2. Description of the assembled reads

№	Sample	Contig length, MBases	Maximal contig length, b. p.	N50, b. p.
1	4B_S2	73	50917	2790
2	12_S1	160	111721	3800
3	D3F	106	855598	9284
4	D4F	237	433763	5677
5	D5F	140	517131	21016
6	D6F	238	544506	5742
7	D11F	46	1671967	7207
8	D12F	147	545374	7999
9	D13F	317	643760	12617
10	DG_S1	208	125246	2621
11	HG550	82	69816	3121

phosphotransferase genes [Kovtun AS, unpublished] could be assigned to certain species. Results of the bioinformatic analysis are presented in Table 5.

DISCUSSION

The *in silico* analysis of 11 gut metagenomes of healthy Russians revealed the presence of aminoglycoside phosphotransferases in 7 metagenomes. Of 21 aph genes previously isolated from the clinical strains of *Acinetobacter baumannii*, *Alcaligenes faecalis*, *Bacillus circulans*, *Burkholderia pseudomallei*, *Campylobacter jejuni*, *Enterococcus faecalis*, *Escherichia coli*, *Enterococcus casseliflavus*, *Enterococcus faecium*, *Legionella pneumophila*, and *Pseudomonas aeruginosa* listed in our aph catalog (Table 3), only 3 were found in the studied samples. Those are: *aph(3'')-Ib*, *aph(3')-IIIa* and *aph(2'')-Ia*. The most frequently occurring gene was *aph(3')-IIIa* (CAA24789) detected in 6 samples. This gene was previously discovered in *E. faecalis* and confers resistance to kanamycin. Gene aph(3'')-Ib (AAA26442) previously isolated from *E. coli* and associated with streptomycin resistance and gene aph(2'')-Ia (AAA26865) previously isolated from *E. faecalis* and associated with tobramycin resistance were observed in only one studied metagenome (Table 3).

Interestingly, the analysis of contigs that harbor aminoglycoside phosphotransferase-encoding genes revealed

Table 3. The catalog of aminoglycoside phosphotransferase-encoding genes of clinically relevant bacterial strains

Gene name	GenBank entry	Bacteria*	Gene location	Aminoglycoside resistance
aac(6')-Ie-aph(2")-Ia	AAA26865	*Enterococcus faecalis*	Chromosome	Tobramycin
aph(2'')-IIa	AAK63040	*Escherichia coli*	Chromosome	Kanamycin, gentamicin
aph(2'')-IIIa	AAB49832	*Enterococcus gallinarum*	Chromosome	Gentamicin
aph(2'')-IVa	AAC14693	*Enterococcus casseliflavus*	Chromosome	Gentamicin
aph(2'')-Ie	AAW59417	*Enterococcus faecium*	Chromosome	Gentamicin
aph(3')-Ia	CAA23656	*Escherichia coli*	Transposon Tn903	Kanamycin
aph(3')-Ib	AIL00451	*Pseudomonas aeruginosa*	Chromosome	
aph(3')-IIa	CAA23892	*Escherichia coli*	Transposon Tn5	Neomycin
aph(3')-IIb	AAG07506	*Pseudomonas aeruginosa*	Chromosome	Kanamycin, neomycin, butirosin, seldomycin
aph(3')-IIIa	CAA24789	*Enterococcus faecalis*	Chromosome	Kanamycin
aph(3')-IVa	P00553	*Bacillus circulans*	Transposons Tn5 and Tn903	Kanamycin, neomycin
aph(3')-VIa	CAA30578	*Acinetobacter baumannii*	Chromosome	Kanamycin, amikacin
aph(3')-VIb	CAF29483	*Alcaligenes faecalis*	Transposon Tn5393	Kanamycin, streptomycin, amikacin
aph(3')-VIIa	P14508	*Campylobacter jejuni*	Chromosome	Kanamycin, neomycin
aph(3')-VIIIa	P14509	*Escherichia coli*	Plasmid RP4	Kanamycin, neomycin
aph(3'')-Ib	AAA26442	*Escherichia coli*	Plasmid RSF1010	Streptomycin
aph(4)-Ia	P00557	*Escherichia coli*	Plasmid pJR225	Hygromycin
aph(4)-Ib	CAA52372	*Burkholderia pseudomallei*	Chromosome	Hygromycin
aph(6)-Ic	CAA25854	*Escherichia coli*	Transposon Tn5	Streptomycin
aph(6)-Id	AAA26443	*Escherichia coli*	Plasmid RSF1010	Streptomycin
aph(9)-Ia	AAB58447	*Legionella pneumophila*	Chromosome	Spectinomycin

Note. * — a microorganism the gene was first isolated from.

Table 4. Aminoglycoside phosphotransferase genes identified in the studied metagenomes

Gene name	Metagenome ID										
	4B_S2	12_S1	D3F	D4F	D5F	D6F	D11F	D12F	D13F	DG_S1	HG550
aph(2'')-Ia	–	–	–	–	–	–	–	+	–	–	–
aph(3'')-Ib	–	–	–	–	+	–	–	–	–	–	–
aph(3')-IIIa	–	–	+	+	-	+	–	+	+	–	+

Table 5. Diversity of species in the studied metagenomes with identified aminoglycoside phosphotransferase genes

Metagenome	Contig length, b.p.	*aph(2'')-Ia*	*aph(3'')-Ib*	*aph(3')-IIIa*
D3F	3389	–	–	*Enterococcus faecium*
D4F	6439	–	–	*Ruminococcus obeum*
D5F	1422	–	*Escherichia coli*	–
D6F	979	–	–	*Enterococcus faecium*
D12F	5607 (для гена *aph(3')-IIIa*); 4407 (для гена *aph(2'')-Ia*)	*Clostridium difficile*	–	*Roseburia hominis*
D13F	4356	–	–	*Streptococcus pyogenes*
HG550	2242	–	–	*Staphylococcus epidermidis*

the presence of the latter in the genomes of other bacterial species. For example, the *aph(3')-IIIa* gene was detected in a sequence typical for commensal *Ruminococcus obeum* and opportunistic *E. faecium*, *Roseburia hominis*, *Streptococcus pyogenes* and *Staphylococcus epidermidis*, but not for *E. faecalis*. Gene *aph(2'')-Ia* was detected in *Clostridium difficile*, but not in *E. faecalis* (Tables 3, 5). Although this gene was the most prevalent in enterococci in the study [16], we observed it in only one studied sample in the non-enterococcal sequence. Genes *aph(2")-Ia* and *aph(3')-IIIa* were previously reported in methicillin-resistant strains of *Staphylococcus aureus* [17]. However, in the studied Russian metagenomes aph(3')-IIIa was present in the sequence typical for *Staphylococcus epidermidis*, while *aph(3'')-Ib* was detected in *E. coli*.

These results are consistent with the results of comparative analyses conducted worldwide: age, sex and health do not have any significant impact on the antibiotic resistance of the gut microbiota, while the geographic origin does [15]. Rare occurrence and poor diversity of *aph* genes in Russian metagenomes may indicate that gut microbiota composition is specific to a particular region and that individuals whose microbiomes were analyzed in our study rarely resort to aminoglycoside therapies. On the other hand, missing *aph* genes in anaerobic bacteria that dominate the gut microbiota may be explained by the absence of cytochrome-mediated transport [25]. It is also important that the microbiome of a healthy individual harbors opportunistic bacteria carrying *aph* genes.

CONCLUSIONS

Previously isolated from clinical bacterial strains, genes *aph(3'')-Ib*, *aph(3')-IIIa* and *aph(2'')-Ia* were found in 7 microbiota samples of 11 healthy Russians. Gene aph(3')-IIIa prevailed. The genes detected in the samples are carried by opportunistic bacteria: *Enterococcus faecium*, *Roseburia hominis*, *Clostridium difficile*, *Escherichia coli*, *Streptococcus pyogenes*, and *Staphylococcus epidermidis*. Two of them — *E. coli* and *E. faecium* — belong to a group of 12 highly dangerous bacteria, according to the World Health Organization. Therefore, we believe it reasonable to run antibiotic resistance tests on both the causative agent and patient's microbiota before deciding on the antibiotic treatment for patients with bacterial infections.

This work is the first to study the spread of antibiotic resistance genes of the gut microbiota of Russians. Further PCR-based search should be conducted to identify other clinically relevant resistance genes.

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