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REVIEW ARTICLE

Diverse Distribution of Resistomes in the Human and Environmental Microbiomes

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Abstract: The routine therapeutic use of antibiotics has caused resistance genes to be disseminated across microbial populations. In particular, bacterial strains having antibiotic resistance genes are frequently observed in the human microbiome. Moreover, multidrug-resistant pathogens are now widely spread, threatening public health. Such genes are transferred and spread among bacteria even in different environments. Advances in high throughput sequencing technology and computational algorithms have accelerated investigation into antibiotic resistance genes of bacteria. Such studies have revealed that the antibiotic resistance genes are located close to the mobility-associated genes, which promotes their dissemination. An increasing level of information on genomic sequences of resistome should expedite research on drug-resistance in our body and environment, thereby contributing to the development of public health policy. In this review, the high prevalence of antibiotic resistance genes and their exchange in the human and environmental microbiome is discussed with respect to the genomic contents. The relationships among diverse resistomes, related bacterial species, and the antibiotics are reviewed. In addition, recent advances in bioinformatics approaches to investigate such relationships are discussed.

Keywords: Resistome, Microbiome, Antibiotic resistance genes, Metagenomics, Environmental microbiomes, Human microbiome.

1. INTRODUCTION

Antibiotics are produced by microorganisms in their competition for survival against other organisms. Recent studies based on genome and metagenome sequencing data have revealed gene clusters that produce antibiotics. More than three thousand biosynthetic gene clusters producing small molecules of antibacterial activity have been found in the human microbiome [1]. Equally important within this context are bacterial mechanisms to attenuate the effects of antibiotics produced by natural enemies. Indeed, investigation on human microbiomes has revealed a diverse distribution of antibiotic resistance genes in different race populations [2, 3]. Even in the microbiomes that have not been exposed to antibiotics, a wide range of resistance genes is observed [4, 5].

The introduction and widespread use of synthetic antibiotics have saved numerous human and animal lives from bacterial infections. However, it did not take long to observe prevalent resistance after the first antibiotic, penicillin was prescribed to treat bacterial infection in the 1940s [6]. In addition to penicillin-resistant *Pneumococcus*, resistant bacteria such as tetracycline-resistant *Shigella*, methicillin-resistant *Staphylococcus aureus* (MRSA), and gentamicin-resistant *Enterococcus* were subsequently discovered [7].

Recent trends of increasing antibiotics usage continue to elevate the spread of resistant genes among microbes around us. In fact, drug resistance is becoming a major threat to public health. MRSA infection is one of the most serious threats, which causes over ten thousand deaths per year in the U.S. alone [8]. Nowadays, antibiotics are widely used in animal husbandry to promote the health and growth of livestock, which can be a potential threat for spreading drug-resistant genes. For example, after the use of avoparcin was banned in Demark in 1995, the population of vancomycin resistant *E. faecium* was significantly reduced from 72.7% to 5.8% in broilers, and from 20.0% to 6.0% in pigs in just five years [9]. A close association between the antibiotic usage and the prevalence of resistance is becoming evident.

Bacteria acquire antibiotic resistance by mutation of their own genes or by receiving genes from other bacteria through conjugation, transduction, or transformation [10]. An *in vivo* study found 35 mutations in the vancomycin-resistant isolates of *Staphylococcus aureus*, including one insertion and nine deletions, after an extensive treatment of vancomycin and other antibiotics including rifampin and imipenem [11].

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Several mutations are thus involved in rifampin and β-lactam resistance genes. Horizontal gene transfer is another important mechanism to spread resistance genes in bacterial microenvironments. For example, *Bacteroides* plays a central role in distributing resistance genes among microbes in the human colon [12]. An extensive gene transfer was observed among different bacterial species, even among different genera. In particular, the resistant bacteria could be transferred to humans or livestock through farming [13]. An interesting study showed that the appearance of tetQ, one type of tetracycline resistance gene, in *Bacteroides* strains has more than doubled over the three decades [12]. The tetQ variants are highly homologous, showing more than 96% DNA sequence similarity, which results from horizontal gene transfer.

Horizontal transfer through outer membrane vesicle (OMV) was also observed in several studies. One of the pioneering *in vitro* studies showed that OXA-24 carbapenemase gene in *A. baumannii* strains is transferred through OMV to a carbapenem-susceptible *A. baumannii* strain [14]. β-Lactam-susceptible *E. coli* was also reported to obtain the resistance through OMV [15]. Resistance genes are currently found in microorganisms not only in hospitals, but also in environments such as sewage, soil, and drinking water (Fig. **1**). Transferred genes can travel further to different environments through diverse media. A recent metagenomics study reported high acquisition rates of resistance genes for 122 healthy individuals who had made trips to different countries: *e.g.* an increase in the β-lactamase activity from 9% to 37% [16]. The prevalence of resistance genes that are mutated or transferred among the bacterial communities is a significant problem for public health.

The prevalence of resistance genes has also been observed in the beneficial species. For example, *Bifidobacterium* species are beneficial microbes as probiotics. As it turns out, a significant number of *Bifidobacterium* strains have resistance genes such as *tetM*, *tetW*, and *tetO* in their genome [17]. These strains, however, do not show any strong resistance. Another study also found the resistance gene *tetW* from *B. longum* [18]. In particular, tetracycline genes are almost ubiquitous across different human body sites, such as oral, skin, and vagina [19].

In this review article, we provide a comprehensive overview of recent metagenomics studies on the prevalence of antibiotic resistance. We focus primarily on the genomic investigation of the microbiomes from different environments, such as human, water, and soil. In addition, the results of experimental validation and functional screening are provided.

2. MECHANISMS AND PREVALENCE OF ANTIBIOTIC RESISTANCE

Antibiotic activity arises from different mechanisms: inhibition of cell wall synthesis or disruption of lipid membrane integrity (*e.g.* β-lactams and glycopeptide); inhibition of protein synthesis (*e.g.* aminoglycoside and tetracycline); inhibition of DNA replication or disruption of DNA integrity (*e.g.* quinolones and rifampin). β-Lactams possess a four-

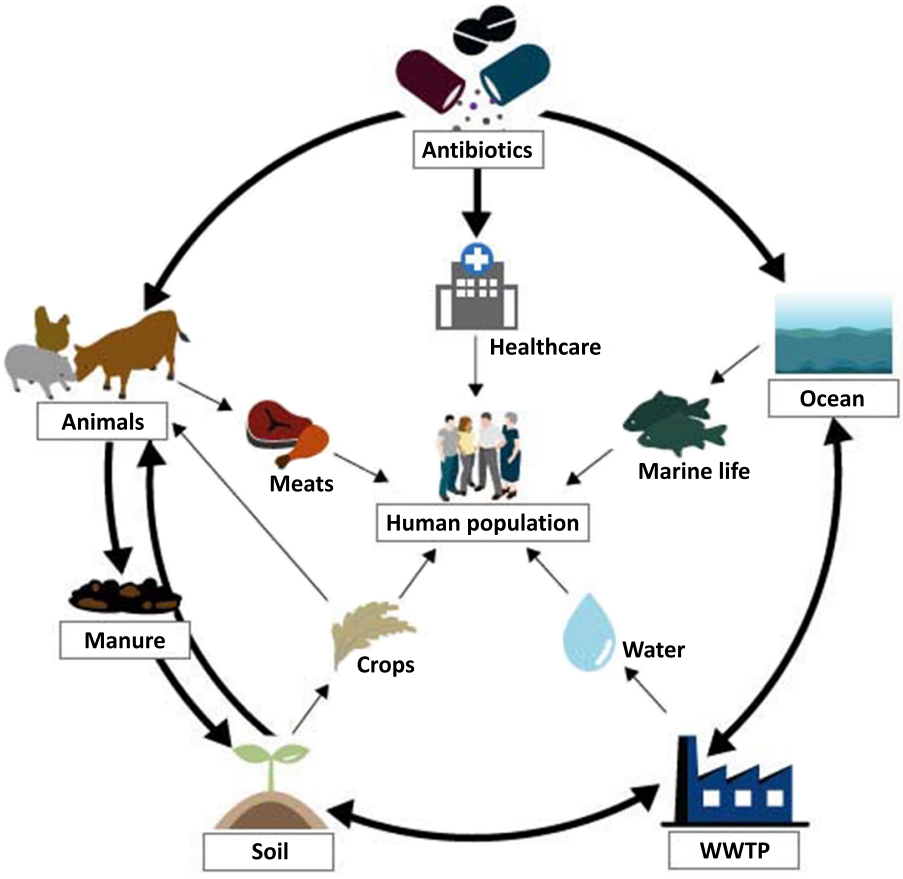

Fig. (1). Overview of wide prevalence and transfer of antibiotic resistance genes in the environment.

atom β-lactam ring, which is the target of β-lactamases that catalyze the cleavage of the bond between the nitrogen atom and the carbonyl group [20]. Since the report on the resistance for penicillin, the first synthetic antibiotic, structural derivatives such as cephalosporin and carbapenem have been developed. Amber classified β-lactamases based on the protein structural information: serine β-lactamases class A, C, and D; metallo-β-lactamases class B [21]. This classification is widely used for screening β-lactamases and comparing their abundance in the microbiome.

The inactivation of tetracycline mainly involves two different mechanisms: ribosomal protection and efflux pump [22]. Enzymatic inactivation is also observed for tet(X) and tet(34). The resistance genes encoding efflux pump mainly belong to the Major Facilitator Superfamily (MFS). Since tetracyclines are broad-spectrum agents for both Gram-positive and Gram-negative bacteria, and are also used for promoting animal growth, their resistance is prevalent. As such, they are responsible for the most abundant resistant genes in animal and human gut [3].

Quinolones and rifampin are inactivated by mutating or modifying their target sites. Quinolones exert antibacterial effects by altering DNA replication through the inhibition of DNA gyrase and topoisomerase IV. The resistance obtained through the enzymatic modification of the target site is well characterized in the *erm* genes involved in erythromycin ribosomal methylation. The macrolides act on their target bacteria by binding to the P sites on the 50S subunit of the bacterial ribosome, thus inhibiting protein biosynthesis. The methyltransferases encoded by *erm* genes methylate the 23S ribosomal RNA at nucleotide A2058, which reduces the binding affinity of the macrolides to the ribosome. More than 30 *erm* genes have been found in over 30 bacterial genera [23].

The overexpression of efflux pumps lowers the intracellular concentration of the antibiotics. There are five families of efflux pumps: (i) the resistance-nodulation-division (RND) family, (ii) the major facilitator superfamily (MFS), (iii) the ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, (iv) the small multidrug resistance (SMR) family, and (v) the multidrug and toxic compound extrusion (MATE) family. These families are classified according to their composition, the number of transmembrane spanning regions, energy sources and substrates, and bacterial efflux pumps [24]. Some efflux pumps, such as multidrug resistance (MDR) efflux pumps, secrete a wide range of antibiotics such as fluoroquinolones, β-lactams, and carbapenems from the cells. In contrast, the *tet* genes for tetracycline in many Gram-negative bacteria, or *mef* genes for macrolides in *S. pyogenes*, *S. pneumoniae* and Gram-positive bacteria are involved in removing antibiotics from the cell in a substrate-specific manner [25].

3. SCREENING ANTIBIOTIC RESISTANCE GENES IN THE MICROBIOME

Culture-based approaches for the identification and characterization of antibiotic resistance genes have limitations. Many antibiotic-resistant bacteria cannot be cultured to characterize the resistance and to reveal the genomic information. Thus to identify the resistance genes in the microbiome, three different culture-independent approaches are often employed: PCR-based screening, functional metagenomics method, and homology-based sequence search.

3.1. PCR and Microarray-Based Screening

PCR and microarray have been extensively used to find resistance genes from the metagenomes [26]. The results, however, might be biased by the choice of the primer sequences. In addition, DNA microarray-based approaches could suffer from cross-talk among different probes, resulting in false predictions. In spite of such limitations, targeted screening for specific resistance genes in a micro-habitat has been widely performed. For example, vancomycin resistance genes were detected in wastewater and surface water in Europe by using a specific primer of *van* genes. Tetracycline resistance genes were also screened in water environments by using PCR-based methods [26].

3.2. Functional Metagenomics Method

To study antibiotic resistance genes without culture and sequencing bias, functional screening has been applied to metagenomic libraries [27, 28, 30] (Fig. **2**). The extracted DNA is sheared to a target size distribution and then cloned into expression vectors. The libraries of cloned DNA are transformed into a heterologous host, generally *E. coli*, and plated on media containing antibiotics. The vector inserts that convey resistance are sequenced, assembled, and annotated.

This method is useful in identifying novel resistance genes in an environment. A pioneering study applying functional metagenomics found 95 resistance genes that show various levels of homology to the known genes in GenBank, ranging from 100% to 40% at the nucleotide level, and from 100% to 20% at the amino acid level [29]. On average, 69.5% nucleotide sequence similarity and 65.3% amino acid sequence similarity were observed.

3.3. Homology-based Sequence Search

Many resistance genes have been sequenced and deposited into the public sequence databases. In recent years, several database systems of resistance genes have been constructed by comprehensively collecting such sequences (Table **1**). Most of the databases provide sequences available to the public, allowing on-line search functions using the BLAST program.

Resistome studies using shotgun metagenome sequencing could successfully describe the global landscape of the resistance gene distribution. Compared with the functional selection, shotgun metagenome sequencing can provide fast, high-throughput profiles of resistomes in the environment. Resistance profiles are analyzed by mapping the sequencing reads, or assembled contigs to the antibiotic resistance gene database. This method, therefore, can only identify previously known antibiotic resistance genes or genes having high homology to the known resistance genes. The search results need further validation to confirm whether the identified genes have actual functions of conferring the resistance to antibiotics.

The Antibiotic Resistance Genes Database (ARDB) is the first database that applies a comprehensive ontology to

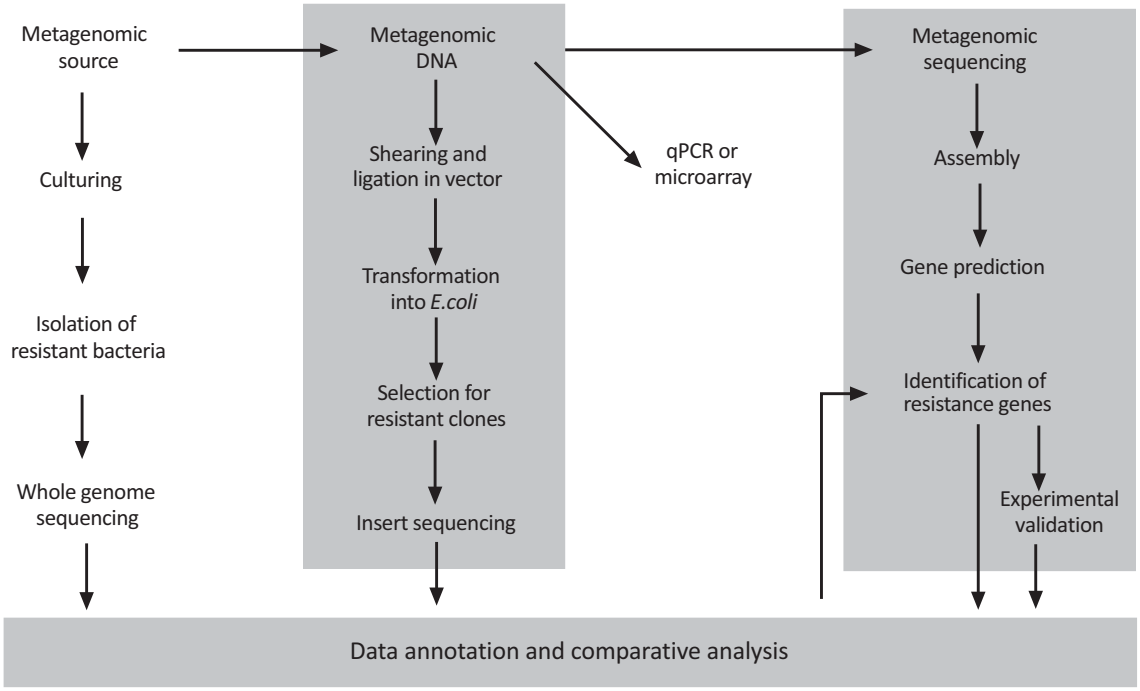

Fig. (2). The process of profiling resistomes based on the metagenomics approach.

Table 1. A list of resistome sequence databases and methods for prediction.

Name	Website	# of Genes	Functions Provided	Sequence Availability	References
ARDB	https://ardb.cbcb.umd.edu	23,137	Homology search against AR database, annotation of ar genes in single genome, browsing information of resistance genes	o	[31]
LacED	http://www.laced.uni-stuttgart.de/	2,399	Blast search	o	
CARD	https://card.mcmaster.ca	2,357	Blast search, Browsing ontology	o	[33, 34]
ResFams	http://www.dantaslab.org/resfams/	170 pHMMs	-	o	[35]
ResFinder	https://cge.cbs.dtu.dk/services/ResFinder/	2,175	Blast search	o	[38]

classify genes based on the resistance mechanisms and their activities [31]. Since the database became available to the public in 2008, it has been extensively used to find antibiotic resistance genes in a single genome as well as in Microbiome. Currently, ARDB includes 23,137 genes, which encompass 380 types, 249 antibiotics, 632 genomes, 1,737 species, and 267 genera. This database can be accessed through the web interface, along with search functions on their own database. In addition to the homology search against resistance gene sequences, resistance-related polymorphism can also be detected in 12 genes such as 16S rRNA and 23S rRNA. The Lactamase Engineering Database (LacED) is a specialized database for the class A and class B of β-lactamases, which was built in 2009 [32]. A total of 483 TEM, 347 SHV, and 597 class B β-lactamases have been collected by parsing NCBI protein database and TEM mutation tables.

These first-generation databases have recently been integrated into the Comprehensive Antibiotic Resistance Database (CARD). CARD incorporates essential information, such as protein structures, mechanisms, and antibiotic targets [33]. Currently, more than 2100 known antibiotic resistance genes, collected from ARDB and Genbank, are classified by using the antibiotic resistance ontology. CARD uses two models, protein homology and protein variant, to detect resistance protein sequences or resistance-conferring mutations. At this point, CARD is one of the most updated resistance gene database systems. In the recently updated CARD, model ontology has been introduced [34].

Homology-based search requires selection of a score threshold for the sequence similarity, which introduces an arbitrary criterion. Hu *et al.* investigated the similarity threshold in homology search to find that 80% cutoff shows the best performance in terms of the precision and recall rates [3]. In order to overcome the limitations of pairwise alignment on remotely homologous resistance genes, the Resfams database of profile Hidden Markov Models (HMMs) for antibiotic resistance-specific gene families was

built [35]. Based on the genes that are used to train the models, there are two different versions. Protein sequences were collected from the CARD database [33], the Lactamase Engineering Database (LacED) [32], and Jacoby and Bush's collection of curated β-lactamase proteins (http://www.lahey.org/Studies/). Those sequences were used to train 123 profile HMMs in addition to 47 profile HMMs from the Pfam [36] and TIGRFam [37], resulting in a total of 170 profile HMMs. These models were evaluated by using resistance gene sequences that were not included in the training dataset. A high precision and recall rates above 98% were shown. Since certain types of resistance genes in CARD are not included, the two databases need to be considered simultaneously for a better performance of searching resistance genes.

Currently, there are several predictors for resistance genes available to the public as online applications. For example, ResFinder performs homology-based sequence search against 2,175 antibiotic resistance gene database [38]. Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) uses a local BLAST program to detect point mutations of antibiotic resistance genes, including both known antibiotic resistance genes and putative antibiotic resistance genes in bacterial genomes [39]. ARG-ANNOT database has 1,689 antibiotic resistance genes curated from Lahey Clinic website, ARGO, MvirDB, ARDB, and ResFinder, along with publications from PubMed.

Due to the increase in antibiotic-resistant bacteria, susceptibility testing is becoming more important. This testing can be done by two different methods: phenotype testing and genotype testing. Currently, phenotype testing is more widely used. The testing, however, could take from 1-2 days to weeks, depending on the bacterial characteristics. To overcome this bottleneck, a few genotype methods have become available. Iqbal *et al.* proposed a method that uses *de Bruijn* graph to represent genomic variations [40]. Graphs are generated from the resistant mutations and genes in the sample, as well as from the reference genome, and resistance-related alleles are detected by comparing the graph topologies. Dantas *et al.* developed a Genotype-Based Antibiotic Susceptibility Prediction (GBASP) algorithm to predict the antibiotic susceptibility using machine learning and rules-based approaches [41]. In the machine learning process of this algorithm, a logistic regression method was trained with the features of resistance genes determined by Resfams and the phenotypic values determined by *in vitro* experiments.

4. DIVERSE DISTRIBUTION OF RESISTOMES IN HUMAN MICROBIOME

During the last decades, the human microbiome has been extensively studied for the compositional diversity with respect to the disease status, diet, and lifestyle [42-44]. Moreover, the human gut has received increasing attention as a reservoir for antibiotic resistance genes, providing answers to the questions regarding the diversity and the abundance of resistance genes in the normal flora of human microbiome [2, 3, 45, 46]. Many studies revealed that antibiotic treatments directly affect the diversity of bacterial composition [47, 48]. An increase of the resistance genes in the microbiome was also observed after antibiotic treatments [49, 50].

In the early 2010s, two international human microbiome consortiums, MetaHIT and HMP, made publicly available human gut microbiome data of European, American, and Chinese subjects [43, 44]. With the advances in high throughput sequencing of the human microbiome, new methods have been developed to screen resistance genes for resistome studies in an untargeted and unbiased manner (Table **2**). As pioneering studies, Forslund *et al.* and Hu *et al.* published large-scale resistome screening results using gut microbiomes of diverse populations [2, 3]. Both studies used quite similar computational methods of homology-based screening against ARDB.

Specifically, Forslund *et al.* screened 252 fecal microbiome from three different populations of 71 Danish, 39 Spanish, and 142 American to determine the antibiotic resistance potential of 50 antibiotic classes and subclasses in the human gut microbiome [2]. The results also showed that the Spanish gut microbiome had more abundant resistance genes than those from Danish or American. According to the statistics of antibiotics use in humans, resistance potential of samples from such countries is positively correlated with antibiotics exposure.

Hu *et al.* screened 162 fecal microbiomes, including 124 from the MetaHIT studies (85 Danish and 39 Spanish) and an additional 38 Chinese individuals [3]. The Chinese population showed a quite comparable ratio of resistance genes with the Spanish population, which is significantly higher than that of the Danish population. This finding is consistent with the results of Forslund *et al.* [2]. Similarity cutoff is critical in the homology-based search. A total of nine types, *ant6ia*, *bacA*, *vanRA*, *vanRG*, *tet32*, *tet40*, *tetO*, *tetQ*, and *tetW*, were found in all 162 samples.

In addition to the large-scale gut microbiome from Europe and America, more diverse microbiome from the Asian population was screened by Ghosh *et al.* [46]. In an effort to build an integrated gene catalog from global microbiome, Yang *et al.* screened more comprehensive population-level gut resistome using 1,267 fecal metagenomes from 139 American, 368 Chinese, 401 Danish, and 359 Spanish samples [51]. Similar diversity and abundance patterns of resistance genes were observed across the continents. It should be noted that this study integrated most of the large-scale human gut microbiome data. As a result, there is some redundancy in the data when compared with previous studies [2, 3]. Vancomycin, followed by macrolide-lincosamide-streptogramin (MLS) and tetracyclines resistance genes were the most abundant in all of the four countries.

Resistome screening using functional metagenomics was also applied to the oral microbiome of two healthy individuals to identify 95 novel resistance genes [29]. In this study, the authors additionally screened cultured aerobic isolates from the gut microbiota and found that almost half of the resistance genes were perfectly identical to the resistance genes from human pathogens. Clemente *et al.* also screened oral samples of four uncontacted native Americans who lived in an isolated Yanomani village [5]. Twelve functional antibiotic resistance genes were found in these oral metagenomic libraries. Despite no known exposure to antibiotics, they harbored resistant genes such as penicillin-binding proteins, chloramphenicol acetyltransferase, and transporters. In

Table 2. Summary of studies characterizing human resistomes through metagenomic sequencing.

Body Site	# of Samples	Age	Country	Dominant Resistance Determinant	References
Gut	252 fecal samples	Adult	71 Danish, 39 Spanish, and 142 American	Tetracycline, bacitracin, β-lactam, macrolide-lincosamide- streptogramin	[2]
Gut	162 fecal samples	Adult	85 Danish, 39 Spanish, and 38 Chinese	Aminoglycoside, bacitracin, tetracycline, glycopeptide	[3]
Gut	275 fecal samples	Adult	92 American, 85 Danish, 39 Spanish, 30 Chinese, 13 Japanese, 8 French, 6 Italian, and 2 Indian	Tetracycline, bacitracin, vancomycin	[46]
Gut	1,267 fecal samples	Adult	139 American, 368 Chinese, 401 Danish, and 359 Spanish	Tetracycline, glycopeptide, macrolide-lincosamide- streptogramin, β-lactam	[51]
Gut	70 fecal samples (35 students before and after travel from Sweden to the Indian peninsula or to Central Africa)	Adult	35 Swedish	Aminoglycoside, β-lactam, sulfonamide, tetracycline, trimethoprim (significant changes after travel)	[70]
Gut	401 fecal samples collected longitudinally from 84 infants	Infant	84 American	β-Lactam, amphenicols, tetracyclines	[71]
Gut	5 fecal samples taken from a single ICU patient during and after an ICU stay	Adult	1 Dutch	Aminoglycoside, macrolide, tetracycline	[72]
Gut	19 fecal samples taken from four individuals before, during and after the AB course	Adult	4 German	Aminoglycoside, bacitracin, tetracycline	[73]
Skin	291 skin samples	Adult	15 American	β-Lactam, aminoglycoside, quinolone	[74]

a comparison of such genes with the industrialized control of Puerto Rican individuals, the resistomes of antibiotic-naïve and industrialized individuals were shown to be shared despite the different history of antibiotic exposures.

It is yet to be understood how antibiotic resistance develops. Several studies investigated gut resistome during the early years of life, and their relations with mothers and infants [52-54]. Moore *et al.* characterized the gut resistomes of three healthy twin pairs at three-time points over a period of a year and their mothers at the delivery time by using functional metagenomic selections [52]. They found that healthy 1-2 month-old infants' gut microbiota harbor diverse resistomes distinct from the maternal resistome. Moreover, the gut resistomes of twin infants were more similar to each other than to those of their mothers or unrelated infants. This finding suggests that resistome development in early life may mainly be shaped by family-specific shared environmental factors, not by the maternal gut resistome. In contrast, several groups have focused on the sharing of antibiotic resistance between mother and newborn baby. De Vries *et al.* showed that tetracycline resistance is observed both in mother and one-month old newborn, despite differences in the genes and source organisms [53]. Zhang *et al.* also found that resistance genes are partially shared between infants and mothers, and *tet* genes are the major resistance genes in infants [54]. In order to clarify the process of resistome development in the infant gut microbiome, longitudinal analyses of gut resistomes within a larger twin-family cohort are needed.

Overall, vancomycin, macrolide-lincosamide-streptogramin (MLS), and tetracyclines resistance genes are the most abundant in human gut microbiome [2, 3]. The broad distribution of these resistance genes in human microbiomes might be causally related to antibiotic use in farm animals [55].

5. RESISTOMES IN THE ENVIRONMENTS

Antibiotic-producing bacteria are abundant in nature. In particular, the soil is a reservoir of antibiotic resistance genes. The resistance genes in soil microbiome are important to human health, since humans are constantly exposed to the soil as a living environment as well as a basis for food production. For the soil microbiome, *de novo* approaches, such as functional selection and shotgun sequencing, have been applied to find novel resistance genes [56]. In addition to identifying known resistance genes, finding novel resistance genes that have evolved in nature is very important in soil environment [57] (Table **3**).

Similar to the human resistome, soil bacteria could harbor resistance genes even without any exposure to man-made

Table 3. Summary of studies characterizing environmental resistomes through functional metagenomics.

Environment	# of Clones (Bases)	# of Resistant Clones/ARs Sequenced	Resistance Gene	References
Soil (Alaskan)	714,000 (12.4Gb)	14	β-Lactamase	[57]
Soil (Apple orchard)	446,000 (13 Gb)	13	β-Lactamase, aminoglycoside acetyltransferases, efflux pump	[75]
Soil (Urban environment)	1,400,000 (2.8 Gb)	39	Aminoglycoside acetyltransferases, rifampin ADP-ribosyltransferases, dihydrofolate reductases, transporters	[58]
Soil (Agricultural and grassland)	(13.8 Gb)	2895	β-Lactamase, transporters, Aminoglycoside acetyltransferases	[59]
Soil (Agricultural)	80,000	45	aminoglycoside acetyltransferase, aminoglycoside 6-adenyltransferase, ADP-ribosyl transferase, ribosome protection protein, transporters	[76]
Water (Agro-industrial wastewater)	200,000	1	Multidrug resistance protein from Bcr/CflA subfamily	[77]
Water (WWTP)	7,618	1	β-Lactamase	[65]
Activated sludge	(1.85 Gb)	9	O-Methyltransferase, β-Lactamase, aminoglycoside phosphotransferase	[78]
Activated sludge	400,000 (800 Mb)	79	β-Lactamase, Aminoglycoside phosphotransferase, dihydrofolate reductase, rifampin ADP-ribosyl transferase	[64]

antibiotics. Microbes compete by producing antibiotic agents against each other; resistance could arise as a consequence of such a natural process. By using functional metagenomics, Allen *et al.* determined resistance genes to β-lactam antibiotics from the Alaskan soil that has no known exposure to antibiotics [57]. A single gene was found to contain the full length of both class C and D β-lactamases, which is the first bifunctional β-lactamase. Moreover, evolutionary analyses showed that the Alaskan β-lactamases are distinct from previously reported β-lactamases. It was suggested that diverse resistance genes have evolved even in the absence of selective pressure, and that β-lactamases of Alaskan soil might be closely related to ancestral enzymes.

The soil collected from an urban environment was investigated to find genes conferring resistance to one of the six antibiotics of kanamycin, gentamicin, rifampin, trimethoprim, chloramphenicol, and tetracycline [58]. The metagenomic library containing 2.8 Gb of DNA was constructed and screened to identify 39 clones that carry antibiotics resistance genes of aminoglycoside acetyltransferases, rifampin ADP-ribosyltransferases, dihydrofolate reductases, and transporters. As observed in other studies, many novel resistance genes were found without any homology with the known genes.

Forsberg *et al.* investigated 18 agricultural and grassland soils to find a total of 2,895 antibiotic resistance genes by using functional metagenomic selection method [59]. The resistance genes found had only 61.1% amino acid identity on average to the entries in the NCBI protein database. β-Lactamase was most commonly observed among the genes assigned to the known antibiotic resistance gene functions. While the soil bacterial community composition showed significant correlations with soil resistomes, the mobility-related genes were rarely co-localized with antibiotic resistance genes. This finding implies that bacterial composition is a more significant factor in determining the diversity of resistome in the soil.

Ocean and river are another reservoirs of antibiotic resistance genes. By using functional metagenomics approach with ampicillin, tetracycline, sulfadimethoxine, and nitrofurantoin, it was found that genes resistant to nitrofurantoin and tetracycline are the most abundant in multiple marine sites, which might be the result of antibiotic effluents from terrestrial sources [60]. Similar to the human resistome, tetracycline is the most frequently observed class in water microbiome including ocean and wastewater from livestock [60-62]. Tetracycline-resistant genes, however, have not been found or found at a lower level in the drinking water [62, 63].

Wastewater Treatment Plants (WWTPs) are another potential reservoir contributing to the spread of antibiotic resistance. The continuous input of persistent antibiotic residues excreted from human and animals into WWTPs can lead to the propagation of antibiotics-resistant bacteria. WWTPs were investigated using a combination of functional selections and metagenomic sequencing. In the metagenomic library constructed from a WWTP in Denmark, 79 unique functionally validated resistance genes were identified [64]. The WWTP core resistome showed on average 69.5% nucleotide sequence identity against the GenBank nt database,

and only six genes showed greater than 95% identity. Mapping the sequencing reads of other WWTP facilities to the WWTP core resistome revealed that the WWTP core resistome was shared with multiple facilities. However, in the microbiome of non-WWTP environments such as human gut, cow rumen, permafrost, and aquifer, less than 10% of the WWTP core resistance genes were found, suggesting that the WWTP resistome is disseminated to other microbial communities only to a limited extent.

The functional metagenomics approach has also been applied to find novel antibiotic resistance genes in WWTPs. One such study was performed against ampicillin by Uyaguari *et al.* [65]. The clones conferring resistance include a β-lactamase gene that is 42% identical to a functionally characterized β-lactamase from *Staphylococcus aureus* PC1. This study suggested that a high concentration of the antibiotic resistance genes might be released from WWTP to the coastal ecosystem, and this gene was highly prevalent in the environments surrounding the facility, even though the number of microbes and antibiotic resistance genes is reduced through the treatments.

Resistance genes have been screened for four years in the Shatin WWTP of Hong Kong [66]. The most abundant resistance genes were aminoglycoside (13-24%), tetracycline (11-23%), and sulfonamide (9.5~14%). The average daily mass flows of tetracycline in the wastewater are higher in winter than in summer, which is in agreement with the metagenome data. Gao *et al.* also observed the distribution of a similar set of resistance genes in the WWTP in Michigan, USA [67]. A correlation was observed for tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant.

6. AR GENES SHARED AMONG HUMAN, ANIMALS, AND ENVIRONMENT

Since human, animals, and microbes share the same environment, resistance genes could be transferred in the microenvironment and distributed to different environments. Several resistome studies used functional metagenomics approaches or shotgun metagenome sequencing for the microbiomes existing in different environments.

One of the most comprehensive resistome studies investigated multiple environments, including water, soil, sediments, wastewater biofilm, and feces by using shotgun metagenome sequencing [62]. The feces and wastewater from livestock farm had the highest level of total resistance gene abundance. For animal fecal resistomes, antibiotic resistance genes were enriched more in adult chickens than in chicks. An opposite trend was observed in pigs. The resistome compositions could be correlated with the environment type. Human resistomes were more similar to the pig resistomes than chicken resistomes. In the network analysis, *tetM* and aminoglycoside resistance genes were identified as the hubs of the antibiotic resistance gene modules.

Gibson *et al.* [35] performed an integrated study to find the environment-dependent composition of resistance genes by using multidrug-resistant cultured soil isolates [68], soil microbiota [59], and pediatric gut microbiota [69]. In particular, the soil and human gut resistomes showed significantly different distributions of β-lactamase and tetracycline resistance functions. Combined with the PCA analysis, network analysis using the bipartite graph of samples and resistance functions as nodes revealed environment-specific resistance functions. Tetracycline resistance functions are mainly encoded by different types of genes in soil and human-associated bacteria: Major Facilitator Superfamily (MFS) efflux pumps for soil, and ribosomal protection genes for the human microbiome. For β-lactamase, class A and C are frequently associated with the human gut, whereas class B with the soil. This study suggests that antibiotic resistance functions are constrained by the environment.

Direct or indirect transfer of antibiotic resistance genes among animal, human, and soil have been studied. A total of 864 publicly available metagenomes from human, animals, and external environments were analyzed to find a high potential for the transmission of antibiotic resistance genes [44]. Among 13 investigated environments, pharmaceutically polluted environments had the highest abundance of antibiotic resistance genes. The air samples from Beijing smog harbored the highest diversity. In the human gut, oral, and urogenital samples, as well as in the animal-associated samples, tetracycline resistance genes were the most abundant. In contrast, in the external environments such as sediments, water, soil, and mine, β-lactam resistance genes were highly abundant. According to the principal component analysis based on resistome profiles, human and animal samples clustered together; all other environmental samples except the Beijing smog sample clustered together. This study suggests the potential of transmission. Resistance genes are prevalent in the environment, and such genes are capable of transferring with the help of mobility-related genes.

Overall, these studies suggest that most resistance genes are specific to the environments, while certain resistance genes share high sequence homology among different environments. This observation implies that the resistance genes in one environment could be moved to other environments as a result of horizontal gene transfer among bacterial species. It has also been observed that the abundance of resistance gene types correlates with the bacterial composition in an environment.

DISCUSSION AND CONCLUSION

Recent advances in metagenomics studies have allowed us to investigate the resistome to understand the prevalence and diversity of antibiotic resistance in different environments. Such metagenome sequencing-based approach has the advantage of unbiased and comprehensive profiling, compared with classical PCR-based approaches. The widespread resistance genes that are mutated or transferred among the bacterial communities threaten our public health. Resistance genes have been observed not only in the hospitals, but also in environments such as sewage, soil, and drinking water. A subset of genes from different environments share a high similarity, implying that the resistance genes have been transferred and traveled across different environments. The next-generation resistome studies are expected to empower our surveillance and proactive remedy against the risk of resistance spread.

More intelligent genomics and bioinformatics methods need to be developed to find antibiotic resistance genes with higher accuracy. Two recently developed methods, *i.e.* metagenomic library-based functional selection and shotgun metagenome sequencing, provide complementary approaches to find novel antibiotic resistance genes and to screen putative resistance genes in a comprehensive manner. Even though the overall trends are consistent among the studies, different screening and profiling methods using various thresholds make it difficult for direct comparisons with respect to the concentration of resistance genes among different samples and environments. Furthermore, if the predicted genes are validated to be functional in antibiotic resistance, they should help better define and classify resistomes of immediate relevance for future therapeutics.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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