Monitoring and Evaluation of Antibiotic Resistance Genes in Three Rivers in Northeast China

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Research

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Abstract

Background: Antibiotic resistance genes (ARGs) have become an important public health problem. In this study, we used metagenomic sequencing to analyze the composition of ARGs in certain original habitats of northeast China, comprising three different rivers and riverbank soils of the Heilongjiang River, Tumen River, and Yalu River.

Results: Twenty types of ARG were detected in every water sample. The major ARGs were multidrug resistance genes, at approximately 0.5 copies/16s rRNA, accounting for 57.5% of the total ARG abundance. The abundance of multidrug, bacitracin, beta-lactam, macrolide-lincosamide-streptogramin, sulfonamide, fosmidomycin, and polymyxin resistance genes covered 96.9% of the total ARG abundance. No significant ecological boundary of ARG diversity was observed. The compositions of the resistance genes in the three rivers were very similar to each other, and 92.1% of ARG subtypes were shared by all water samples. Except for vancomycin resistance genes, almost all ARGs in riverbank soils were detected in the river water. About 31.05% ARGs were carried by *Pseudomonas*. Opportunistic pathogenic bacteria carrying resistance genes were mainly related to diarrhea and respiratory infections. Multidrug and beta-lactam resistance genes correlated positively with mobile genetic elements (MGEs), indicating a potential risk of diffusion.

Conclusions: The composition of ARGs in three different rivers was similar, indicating that climate played an important role in ARG occurrence. ARG subtypes in river water were almost completely the same as those in riverbank soil. ARGs had no significant geographical distribution characteristics. Many ARGs were carried by human pathogenic bacteria related to human diarrhea and respiratory infections, such as *Pseudomonas aeruginosa* and *Aeromonas caviae*. In general, our results provide a valuable dataset of river water ARG distribution in northeast China. The related ecological geography distribution characteristics should be further explored.

1. Introduction

Antibiotics to treat and prevent bacterial infections are widely used in animal husbandry and aquaculture (Marti et al. 2014). Studies have shown that with the increased use of antibiotics, the number and type of antibiotics in the environment have gradually increased (Zhou et al. 2011). Under the pressure of antibiotic selection, a large number of antibiotic-resistant bacteria (ARB) have appeared, and their antibiotic resistance genes (ARGs) have been rapidly transmitted via horizontal transfer (Aminov 2011). There are also many pollutants in the environment that further promote the horizontal transfer of resistance genes, such as heavy metals (Lin et al. 2019) and nanomaterials (Qiu et al. 2012). Ultimately, ARGs are imported into the water ecosystem. For example, urban sewage (Cui et al. 2020), hospital water (Dias et al. 2020), and aquaculture (Liu et al. 2020) result in the discharge of a large number of ARGs into natural water bodies, making rivers and lakes an important location for ARG exchange and transmission. The close relationship between water and human activities, means that ARGs and ARB in water are a potential threat to human health. Therefore, determining the distribution of ARGs in natural water is

important to evaluate this health risk. Surface water, especially rivers, is an important medium for many material exchanges in ecological niches, including ARGs, such as urban sewage (Yuan et al. 2019), feces (Chen et al. 2019), soil (Zhang et al. 2019), and sediment (Cheng et al. 2020). Rivers flow through many cities, and ARGs in river water will be widely spread in their watersheds. It has been reported that the river environment is easily affected by human activities and has become the most important repository of ARGs. As water sources and important aquaculture environments, rivers have strong influence on public health. Understanding the occurrence, diffusion, and fate of ARGs in rivers are key to controlling the pollution and diffusion of ARGs.

Bacterial antibiotic resistance is inherent in the natural environment, and antibiotic secretion and resistance is an important competition mechanism of the microbial community (Baltz 2008, Newman et al. 2003). At present, a large amount of ARGs have been discharged into rivers by human activities; therefore, it may be difficult to determine the ARG baseline profiles. Nevertheless, an alternative approach would be to study ARGs in a pristine environment. In northeast China, few people live around the Heilongjiang River, Tumen River, and Yalu River. Most cities have less than 250 thousand people, and the area lacks large cities, sewage treatment plants, and hospitals. Characterizing mobile genetic elements (MGEs) and ARGs could be helpful to understand the diffusion potential of ARGs in the initial stage.

In China, there have been numerous investigations into ARGs in the Yangtze River (Wang et al. 2019, Yang et al. 2017, Zhang et al. 2020b), Yellow River (Lu et al. 2018, Shi et al. 2019), and Pearl River (Chen et al. 2015, Li et al. 2018); however, there have been none concerning ARGs in the Heilongjiang River. Compared with other rivers, the Heilongjiang River is located in a colder climate with high humus levels, and thus may have a unique microbial community structure and ARG subtypes. There have been few reports on the distribution characteristics of river ARGs under a low temperature climate. The Heilongjiang River, Tumen River, and Yalu River are located in three different provinces in northeast China, separated by large geographical distances. It is difficult to associate the differences in ARGs with ecosystem functions based on an analysis of the composition of ARGs (Louca et al. 2016). Biogeographical methods are a better way to describe the relationship between microbial communities and geographical characteristics (Berendonk et al. 2015). Some studies found no significant correlation between the distribution of microbial communities and geographical space, indicating that the spread of these microbial communities might not be limited (Angermeyer et al. 2016). However, other studies reported the characteristics of the geographical distribution of microbial communities (Liu et al. 2018b). Currently, there is no consensus on the geographical distribution characteristics of ARGs.

In the present study, ARGs in three different rivers and riverbank soils in certain original habitats of northeast China were investigated using metagenomic analysis, with the following aims: (1) To understand the composition and geographical distribution characteristics of river ARGs in this area, (2) to explore the potential interaction of ARGs in river water and riverbank soil, and (3) to evaluate the potential health risks and horizontal transfer possibilities caused by ARGs in northeast China. This large sequencing dataset for river water and riverbank soil ARGs will help to gain an understanding of the

distribution characteristics of ARGs in northeast China and will clarify the original state of antibiotic resistance in rivers that have been little affected by human activities.

2. Materials And Methods

2.1. Sampling and DNA extraction

Water samples were collected in September 2019 at 12 points in total in the Heilongjiang River, Tumen River, and Yalu River (Fig. 1). Three subsamples were collected at every point. Five liters of water were collected in glass bottles for DNA extraction. Water samples (1 L) were filtered through a 0.45 μm membrane (39 mm diameter). The membranes were washed with phosphate-buffered saline (PBS)-EDTA (1% EDTA) and centrifuged to collect the precipitate for DNA extraction. Eleven soil samples were collected in the riverbank of the water sampling site, 1 sample for each point except for FY, because the FY sample site was sandy with very low microbial abundance. According to the manufacturer's protocol, total genomic DNA extraction was performed using a TIANamp DNA Kit for Soil (Tiangen Biotech, Beijing, China). DNA quality was evaluated using gel electrophoresis (1% agarose). DNA samples were stored at −80 ℃ until sequencing.

2.2. Metagenomic sequencing and analysis

A total of 1 μg DNA per sample was used to generate sequencing libraries of about 350 bp (NEBNext® Ultra™ DNA Library Prep Kit, NEB, Ipswich, MA, USA). The libraries were paired-end sequenced on the Illumina HiSeq platform (Illumina, San Diego, CA, USA). Library construction and sequencing were completed by Novogene (Beijing, China).

2.3 ARGs-OAP operation

ARG gene copy numbers were quantified using the antibiotic resistance genesonline analysis pipeline (ARGs-OAP) operation(Yin et al. 2018). ARGs-OAP can be accessed through http://smile.hku.hk/SARGs. This pipeline is based on the SARG v2.0 database, which contains sequences from the CARD and ARDB databases, as well as the latest protein dataset from the NCBI-NR database.

2.4 HPB and MGE annotation of ARG-carrying genes and ORFs

The predicted open reading frame (ORF) sequences were searched against ARGs-OAP v2.2 database for ARG-like ORF identification using DIAMOND blastp, with a cut-off e-value of $1e^{-5}$ and an identity cut-off values 45. An ORF sequence was considered to be an ARG-like ORF if its best hit alignment to ARG sequences was under a cutoff of ≥ 45% query coverage. The ARG-like ORF sequences were then classified according to the ARGs-OAP v2.2 database.

Mobile genetic elements (MGEs) were identified using BLASTP to compare the ARGcarrying genes to the A CLAssification of Mobile genetic Elements (ACLAME) amino acid database for plasmids using an E-

value ≤ 10^{-5} and a cutoff of ≥ 50% query coverage. The ISfinder database (Siguier, 2006) was used to find insertion sequences (ISs) with an E-value ≤ 10^{-5} and a cutoff of ≥ 45% query coverage. In addition, searching against the IntegrALL nucleotide database (Moura et al. 2009) was performed to find integrons, with an E-value ≤ 10^{-5} and a cutoff of ≥ 45% query coverage.

Meanwhile, ARG-carrying genes were compared to the human bacterial pathogens (HBPs) genome database using BLASTP with an E-value ≤ 10^{-10} and a cutoff of ≥ 45% query coverage. Each sequence may have multiple aligned results; therefore, for the final alignment results of each sequence, we chose the result with the best hit score as the accurate annotation information.

2.5 Ecological Boundaries Analysis

To research how ARGs change according to latitude and longitude, we used the split moving-window analysis of ecological differentiation, which was achieved using the R package EcolUtils (https://github.com/GuillemSalazar/EcolUtils). The analysis was conducted with ARG type abundance or ARG subtype abundance, and the latitude or longitude of each site. The obtained analysis result was used for plotting using the R (version 3.5.1) package ggplot2. In the split moving-window analysis, we selected Bray-Curtis dissimilarity, set the windows size as 8, and the probabilities for confidence interval was from 0.025 to 0.975. Thus, we could determine the ecological boundary center based on the ARG composition.

2.6 Network Analysis

ARG subtypes and the genus network was created using the following method. A correlation matrix was constructed with ARG and metagenomics data to explore the potential correlations of ARG–ARG, ARG–genus, and genus–genus by calculating all pairwise Spearman's correlation coefficients (ñ) among ARG subtypes and taxonomy (at the genus level) that occurred in at least 70% of samples, using the R (v3.4.0) package psych. A correlation between two nodes was regarded as statistically significant if r ≥ 0.85 and P was ≤ 0.01, which was adjusted using the Benjamini–Hochberg method. Then, the correlation between two nodes was termed the edge file and we added a description to the node file comprising the phylum taxonomy at the genus level and the ARG type or ARG subtype. The network analysis was visualized using the interactive platform of Gephi (v 0.9.2)(Bastian et al. 2009).

The ARG subtypes and MGE network was created using a similar method. A correlation matrix was constructed with ARGs and MGEs to explore the potential correlations of ARG–ARG, ARG–MGE, and MGE–MGE by calculating all pairwise Spearman's correlation coefficients (ñ) among ARG subtypes and MGEs that occurred in at least 70% of samples using the R (v3.4.0) package psych. A correlation between two nodes was regarded as statistically significant if r ≥ 0.9 and P was ≤ 0.01, which was adjusted using the Benjamini–Hochberg method. Then, the correlation between two nodes was termed the edge file and we added a description to the node file comprising the MGEs and the ARG type or ARG subtype. The network analysis was visualized using the interactive platform of Gephi (v 0.9.2).

3. Results

3.1 Diversity and abundance of ARGs and the bacterial community in different rivers

In total, 21 types of ARG were detected in water samples from Heilongjiang River (HLJ), Yalu River (YLJ), and Tumen River (TMJ), as shown in Table S1. Twenty types of ARG were present in all water samples, and bleomycin resistance genes were only detected in a few samples of YLJ and HLJ. The top 10 types of ARG (Fig. 2) were multidrug resistance, bacitracin, beta-lactam, unclassified, aminoglycoside, tetracycline, macrolide-lincosamide-streptogramin (MLS), sulfonamide, fosmidomycin, and polymyxin, covering 96.9% of the ARG abundance. Multidrug resistance genes accounted for 57.5% of the ARGs. We detected approximately 790 ARG subtypes. The top 10 subtypes were *bacA*, *macB*, *acrB*, *mdtB*, *mexF*, *mexT*, *mexW*, multidrug_transporter genes, *ompR*, and cAMP-regulatory protein genes. The subtype with the highest abundance was *bacA*, at about 0.09–0.14 copies/16s rRNA among all ARGs, resulting in bacitracin resistance genes accounting for a large proportion of the total. Multidrug resistance genes had many subtypes with high abundance, and 7 of the top 10 subtypes were multidrug resistance genes, meaning that these genes had the highest proportion.

For YLJ, 676 subtypes were detected in total. The top 10 subtypes were *bacA*, multidrug transporter genes, *ompR*, *mexT*, cAMP-regulatory protein genes, *mexF*, *sul1*, *mdtB*, *macB*, and *acrB*, which included bacitracin, multidrug, sulfonamide, and MLS resistance genes. For TMJ, 453 subtypes were detected, and the top 10 subtypes were *bacA*, multidrug transporter genes, *mexF*, *mex*T, *mexW*, *mdtB*, *mexE*, *ompR*, *oprM*, and *oprN*, which included bacitracin and multidrug resistance genes. The ARG subtypes in HLJ were similar to those in YLJ; class C beta-lactamase was more abundant in HLJ than in YLJ and TMJ.

The heatmap in Fig. 3 shows the top 20 genera in river water samples from each sampling site. Among all samples, Proteobacteria was the major bacteria phylum, accounting for more than 70%. *Pseudomonas* was highly abundant in each sample, while their abundance in JA and DD was low. *Myroides*, a representative genus of Bacteroidetes, showed high abundance in CB and JA. The abundance of other genera in different samples were quite different, suggesting different microbial community structures in each sample site.

To understand the variation in ARG subtypes with geographical distribution, the diversity of latitude and longitude was analyzed using ecological boundaries analysis. The abundance of ARGs changed with latitude and longitude (Fig. S1); however, no obvious ecological boundary center was observed.

3.2 Comparison of ARG profiles from rivers and riverbanks

To explore the relationship between river water and nearby soil, soil samples from riverbanks were also analyzed. From 11 soil samples, 22 types and 228 subtypes of ARGs were detected. Fewer ARG subtypes were detected in soil than in river water. The top 10 subtypes in soil were *vanR*, *bacA*, *mex*F, ADP-ribosylating transferase_arr genes, *macB*, *mdtB*, multidrug ABC transporter genes, multidrug transporter

genes, *mdtC*, *cpxR*, and *aac(2')-I*. Compared with the ARGs in river water, the distribution of highly abundant of ARG types in soil were more complicated.

The ARG subtype composition in different sample sites was similar to other sample sites. As shown in Fig. 4, 95 subtypes of ARGs were detected in all river water samples, accounting for 12.0% of all subtypes, but representing approximately 92.1% of the total abundance. Furthermore, 407 ARGs subtypes, accounting for 51.5% of all subtypes, were present only in one or two samples with very low abundance, representing just 2% of the total. For the soil samples, about 22.4% of ARG subtypes were shared by all samples, covering 93.0% of the total abundance. Moreover, 84 ARG subtypes contributed about 0.7% of the total abundance. As for the total abundance of ARG subtypes in soil, few subtypes had high abundance, and many subtypes contributed only a small part of the total abundance. More attention should be focused on ARG subtypes with high abundance to prevent potential human health risks from ARGs.

To explore the potential correlation among ARGs in river water and soil, ARG types in different groups were compared. As shown in Fig. 5, the abundance of ARGs in soil was lower than that in river water. The ARG compositions in river water and soil were similar, with multidrug resistance genes and bacitracin resistance genes representing large proportions in all samples. In soil, vancomycin resistance genes were a characteristic type, with high abundance (up to 25%). Spectinomycin and bleomycin resistance genes were only detected in river water samples with low abundance. Other members of the top 20 ARGs types were common in river water and soil.

To learn more about the similarities and differences of ARGs in river water and soil samples, ARG subtypes were further analyzed. First, 206 ARG subtypes were detected in both river water and soil, covering 90.4% of the ARGs in soil. Second, 32 ARG subtypes were detected in all samples, whether from river water or soil, including *bacA*, class A beta-lactamase genes, *macA*, *macB*, *arnA*, *vanR*, and *mexB*. *BacA*, *mexB*, and other multidrug resistance genes were widely present in sample sites with high abundance. Third, *vanR* was mainly present in soil, accounting small amount in river water. The PCoA plot (Fig. S2) demonstrates that the river water samples were clearly separated from soil samples, and samples from different rivers were closely associated with each other.

3.3 The hosts of ARGs in river water and their potential pathogenicity

After annotation, about 17960 ARGs were identified as being carried by many kinds of bacteria. In all river water samples, *Pseudomonas*, *Aeromonas*, *Comamonas*, *Acidovorax*, *Stenotrophomonas*, *Herbaspirillum*, *Acinetobacter*, *Achromobacter*, *Delftia*, and *Variovorax* accounted 82.66% of bacteria carrying ARGs. *Pseudomonas* frequently carried ARGs, and 60% of ARGs carried by *Pseudomonas* were multidrug resistance genes. *Aeromonas* carried many kinds of ARGs, such as multidrug, beta-lactam, MLS, tetracycline, and other unclassified ARGs. The result for the three different rivers were similar to each other. For HLJ, the top 10 bacterial genera carrying ARGs were almost the same as those in all samples, including genus and ARGs types. The difference was that *Shewanella* carried more ARGs and

Achromobacter carried fewer. For TMJ, *Hydrogenophage* and *Cupriavidus* carried more ARGs than in HLJ and YLJ. In addition, *Enterobacter* carried more ARGs in YLJ, contributing about 2.08% of ARGs.

Pathogenic bacteria carrying ARGs are a direct threat to human health: therefore, we further analyzed the ARGs present in pathogenic bacteria. The top 10 pathogenic bacteria carrying ARGs are shown in Fig. 6B. *Escherichia coli* carried the most ARGs, representing 26.9% of all ARGs. Notably, *Pseudomonas aeruginosa*, *Bacteroides fragilis*, and other common clinical pathogenic strains carried many ARGs. *Pseudomonas aeruginosa* is associated with serious infection, and often carried ARGs, such as chloramphenicol and streptomycin resistance genes. In the present study, *Pseudomonas aeruginosa* carried mainly multidrug resistance genes with active efflux mechanisms, and beta-lactam, fosmidomycin, and unclassified ARGs. The results for the three rivers were similar. In YLJ, *Brucella abortus* carried eight types of ARGs, representing a serious challenge for antibiotic treatment. For TMJ, *Staphylococcus aureus* and *Bordetella bronchiseptica* MO275 carried more ARGs, especially multidrug resistance genes. These water-borne human pathogenic bacteria carrying ARGs are a direct threat to human health. Our data provide some suggestions for treatment after infection in this area.

3.4 Transfer risk and potential hosts of ARGs in river water

Horizontal transfer is the main method by which ARGs diffuse in the environment. To evaluate the risk of ARG transfer, the transfer risk and potential hosts of ARGs should be further analyzed. In the present study, network analysis was used to describe the correlations between ARGs and bacteria, as well as the relationship between ARGs and mobile genetic elements (MGEs), to explain the potential host and transfer risk of ARGs. As shown in Fig. 7A, the modularization index was 0.661, indicating that the ARGbacteria network had a modular structure. Some multidrug resistance genes, like *omp36* and *ompF*, correlated positively with many species of Proteobacteria, indicating that these resistance genes had a high risk of transfer in Proteobacteria. Some MLS ARGs, such as *macA* and *macB*, correlated negatively with certain Proteobacteria and Actinobacteria, indicating that the transfer risk of *macA* and *macB* in these strains was low. The relationships of ARGs and MGEs was analyzed using the same method, as shown in Fig. 7B. Some ARGs, such as *Oxa-12*, correlated positively with many kinds of MGEs, indicating a high risk of horizontal transfer.

4. Discussion

To protect human health, it is necessary to understand the distribution and transfer risk of ARGs. To date, research has concentrated on the geographical distribution of ARGs (Liu et al. 2018a, Yang et al. 2019). In the present study, three important rivers in northeast China, the Heilongjiang River, Tumen River, and Yalu River, were assessed. River water samples and riverbank soil samples were collected to analyze the ARG composition using metagenetic methods. The relative abundance of ARGs in river water was between 7.5×10^{-1} and 1.25×10^{0} per 16s rRNA gene, and that in the riverbank soil was between 1.75×10^{-1} and 2.25×10^{-1} per 16s rRNA gene. The abundance of resistance genes in rivers and soil were similar to the range of ARG abundances reported previously (Li et al. 2015). In terms of composition, multidrug resistance

genes represented about half of the total abundance, which was related to the high expression of efflux pumps (Hiroshi and Jean-Marie 2012). Other types, such as bacitracin, beta-lactam, and tetracycline resistance genes, also showed high abundance. As expected, these dominant ARG types corresponded to the antibiotics that have been used widely for human treatment and livestock breeding. *BacA* is a ubiquitous ancient resistance gene, which has been obtained by many bacteria during the process of evolution, resulting in the high abundance of bacitracin resistance genes. Among the multidrug resistance genes, multidrug efflux pumps are ubiquitous in bacteria, which not only effectively reduce the concentration of antibiotics, but also participate in other processes, such as detoxification of metabolic intermediates, virulence, and signal trafficking (Bao et al. 2016). The presence of a variety of ARGs with efflux function, like *adeF* and *mexF*, lead to a high proportion of multidrug resistance genes. The increase of polymyxin resistance genes is a concern. Polymyxin is termed the last line of defense of human antibiotics treatment, and the increase in polymyxin resistance genes represents a potential threat to human health.

Among the samples from the three rivers, the abundance of ARGs was highest in YLJ river water and soil, and was lowest in TMJ. In previous studies, the abundance and composition of ARGs in water was seriously affected by human activities, which might be one of the main driving forces for the formation of ARG structures in water. The population living along the coast of YLJ is significantly larger than that of TMJ and HLJ, and the urbanization process has been relatively faster, which might be one of the reasons for the high abundance of resistance genes in YLJ. In terms of their composition, ARGs in soil had certain characteristics. Vancomycin resistance genes accounted for a large proportion in soil, but not in river water. Vancomycin is used to treat humans and was originally produced by *Amycolatopsis orientalis*, which was isolated from soil. There are more vancomycin-producing bacteria in soil, thus the abundance of vancomycin resistance genes was very high. Except for vancomycin resistance genes, the composition of the ARGs in soil was very similar to that in river water, and almost all the resistance gene types detected in soil were found in river water. This might be related to the characteristics of the river itself. When the river is in flood, the water level rises, covering part of the riverbank. In this process, the bacteria and resistance genes in the soil and river water are mixed and exchanged. In the dry season, the water level of the river drops and the riverbank is exposed, which reduces the mixing and exchange of bacteria and resistance genes. The samples in this study were collected in September, which is in the dry season of these rivers. The composition and structure of the bacteria and resistance genes in river water and soil are relatively stable in this period, which might explain the similar composition of ARGs detected in this study. This similarity suggested that rivers could obtain ARGs from the riverbank soil, and the soil could act as a reservoir of ARGs, resulting in secondary ARG pollution.

Notably, the composition of ARGs in the three rivers was very similar, regardless of types or subtypes. The Heilongjiang River, Tumen River, and Yalu River flow in different directions and are located in three different provinces. Although TMJ and YLJ come from the same origin, there are great differences in population density and economic development in the regions they flow through. The results of ecological boundary analysis showed that the abundance and diversity of ARGs did not have a clear demarcation with the change of longitude and latitude. We next considered whether the similarity in ARGs was

determined by climate conditions. These three rivers are all located in northeast China with a northern temperate climate, relatively low temperature, and similar precipitation and other conditions. Therefore, we hypothesized that climate played a decisive role in the composition of ARGs. Some ARG data in different niches were reported in previous studies. In four sewage treatment plants in Harbin, tetracycline and sulfonamide resistance genes were detected (Wen et al. 2016). These ARG subtypes were also detected in river water samples with high abundance in this study. In a study on the effect of soil fertilization on ARGs in northeast China (Li et al. 2020), the reported highly abundant ARGs types were also detected as highly abundant in the present study. In particular, the ARG data in Heihe (HH) were similar to our data. In a large-scale survey of ARGs in drinking water (Ma et al. 2017), bacitracin, multidrug, and sulfonamides resistance genes were the top three ARG types in Heilongjiang Province. These results suggested that the composition of ARGs in different niches in northeast China is similar, indicating that climate might be the decisive factor in the occurrence and distribution of ARGs. Currently, there are no large-scale sequencing data on the distribution of ARGs in the natural environment of northeast China, whether in water, soil, or other niches. Therefore, this conjecture needs to be further verified in future studies.

Compared with ARGs in Yangtze River, ARGs abundance in the three rivers was lower, especially super ARGs like *mcr-1* and *NDM-1*. According to a previous study, *mcr-1* and *NDM-1* were about 1.2×10^9copies/L and 4.1×10^6 copies/L in the downstream of Yangtze River(Wang et al. 2019). In contrast, the highest abundance of *mcr-1* was only 4.0 copies/L and *NDM-1* was not detected in the three rivers. For common ARGs, the abundance of 16s rRNA gene, suls(*sul1* and *sul2*), tets(*tetA*, *tetC*, *tetE*, *tetO*, and *tetW*), erms(*ermB* and *ermF*), and qnrs(*qnrB* and *qnrS*) ranged from 2.9×10^8 to 2.4×10^{10} copies/L, 1.1×10^7 to 3.6×10^8 copies/L, 1.5×10^6 to 4.9×10^7 copies/L, 8.2×10^5 to 1.8×10^7 copies/L, and 7.7×10^3 to 1.5×10^6 copies/L, respectively, with their mean concentration were 1.9×10^8, 2.0×10^7, 6.8×10^6, and 4.2×10^5 copies/L, correspondingly(Yang et al. 2020, Zhang et al. 2020a). In this study, these ARGs abundance ranged from 2.1×10^{-3} to 3.6×10^{-2} copies/16s rRNA, 7.6×10^{-4} to 4.4×10^{-2} copies/16s rRNA, 0 to 4.9×10^{-3} copies/16s rRNA, 1.8×10^{-4} to 7.5×10^{-2} copies/16s rRNA. It seems that ARGs in the three rivers were less than that in the Yangtze River. Actually, there were more ARG subtypes detected in river water samples like *sul3* and *sul4*. In result, sulfonamide resistance genes were more than suls, so was others.

To further evaluate the potential threat caused by ARGs, the hosts carrying ARGs were analyzed, especially human pathogenic bacteria. *Pseudomonas* carry many ARGs. *Pseudomonas* is widely distributed in nature and is closely related to human infection, for example, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas putida*. Among *Pseudomonas* species, *Pseudomonas aeruginosa* was the most common and carried more ARGs, among which multidrug resistance genes accounted for 60% of the total. In a previous study, 82% of *Pseudomonas aeruginosa* isolated from hospital sewage treatment plants showed resistance to multiple antibiotics. *Pseudomonas aeruginosa* can cause serious infections, and is very common in nosocomial infections. The presence of different ARGs, especially multidrug resistance genes, makes treatment challenging. Among all samples in this

study, *Escherichia coli* was the pathogen that carried the most ARGs. Worldwide, antibiotic-resistant *E. coli* are widely present in all kinds of niches. Third-generation cephalosporin-resistant *E. coli* was detected in coastal surface waters in England and Wales (Leonard et al. 2015). In Dutch rivers, 27.6% of *E. coli* are resistant to at least one antibiotic (Blaak et al. 2014) and 42% of *E. coli* in the river Seine in France have at least one antibiotic resistance gene (Servais and Passerat 2009). Other antibiotic resistant pathogenic bacteria are related to human diarrhea, such as *Aeromonas caviae* and *Bacteroides fragilis*, and to respiratory infection, like *Bordetella bronchiseptica*. A previous study has shown that humans can acquire antibiotic resistant bacteria from water while performing water sports or other activities (Leonard et al. 2015). The three rivers in this study were closely related to the daily lives of residents; therefore, we should be vigilant concerning the related risk of infection.

To further understand risk of ARG diffusion, it is important to evaluate the potential horizontal transfer of ARGs. MGEs participate in this process directly. The ARGbacteria network showed the potential hosts of ARGs. In the network, *Aeromonas* was predicted to correlate positively with many types ARGs, including beta-lactam, multidrug, tetracycline, and MLS type genes, and experimentally, these ARGs were detected in *Aeromonas*. This showed that the network was an effective way to find potential hosts of ARGs. Positively correlations between different types of ARGs, such as that between the multidrug resistance gene *ompF* and the beta-lactam resistance gene *PBP-1B*, indicated their co-occurrence. Different ARG subtypes seem to be preferred by different hosts. The multidrug resistance gene *ompF* correlated negatively with the Proteobacteria genus *Rhodoferax*, but positively with the genus *Trabulsiella*. In the ARG-MGEs network, many MGEs correlated positively with ARGs, which is consistent with previous studies (Zhao et al. 2019, Zheng et al. 2018). Some ARG subtypes correlated positively with many MGEs, such as *OXA-12* and *mdtH*, indicating a high risk of horizontal transfer.

There are some limitations to this study. In the process of collecting soil samples, only one mixed sample was collected from each sample site. To improve the representativeness of the samples, soil samples were collected every 1 m and mixed for analysis. For groups with fewer sample sites, there might be a great difference between a single sample data and the average, thus a more intensive sampling strategy might be considered in future research. Moreover, the impact of season should be considered. The sampling time of this study was autumn, and the high temperature in summer might promote the growth and reproduction of many kinds of eukaryotes and strongly affect the distribution of bacteria and ARGs. Therefore, our results are only strictly applicable to the time-frame in which the samples were collected, and different results might be obtained if the study was repeated in other seasons.

5. Conclusions

In this study, we investigated ARGs in the original habitats of three rivers in northeast China. Multidrug resistance genes were the major ARG type detected. The composition of ARGs in three different rivers was similar, indicating that climate played an important role in ARG occurrence. ARG subtypes in river water were almost completely the same as those in riverbank soil. ARGs had no significant geographical distribution characteristics. These results suggested that ARGs have the same basic composition in this

area. Many ARGs were carried by human pathogenic bacteria related to human diarrhea and respiratory infections, such as *Pseudomonas aeruginosa* and *Aeromonas caviae*. Furthermore, many multidrug and beta-lactam ARGs correlated significantly and positively with MGEs, indicating the potential risks of ARG horizontal transfer, especially among human pathogenic bacteria. Overall, these results revealed the direct threats to public health caused by ARGs in this area.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Chen Zhao and Chenyu Li completed the manuscript. Xiaoming Wang, Zhuosong Cao, Chao Gao and Sicong Su collected all samples. Bin Xue, Shang Wang and Zhigang Qiu analyzed the data. Jingfeng Wang and Zhiqiang Shen designed this study.

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Figures

Figure 1

Abundance and diversity of ARGs in the sample sites. Pie charts show the top three ARG types. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

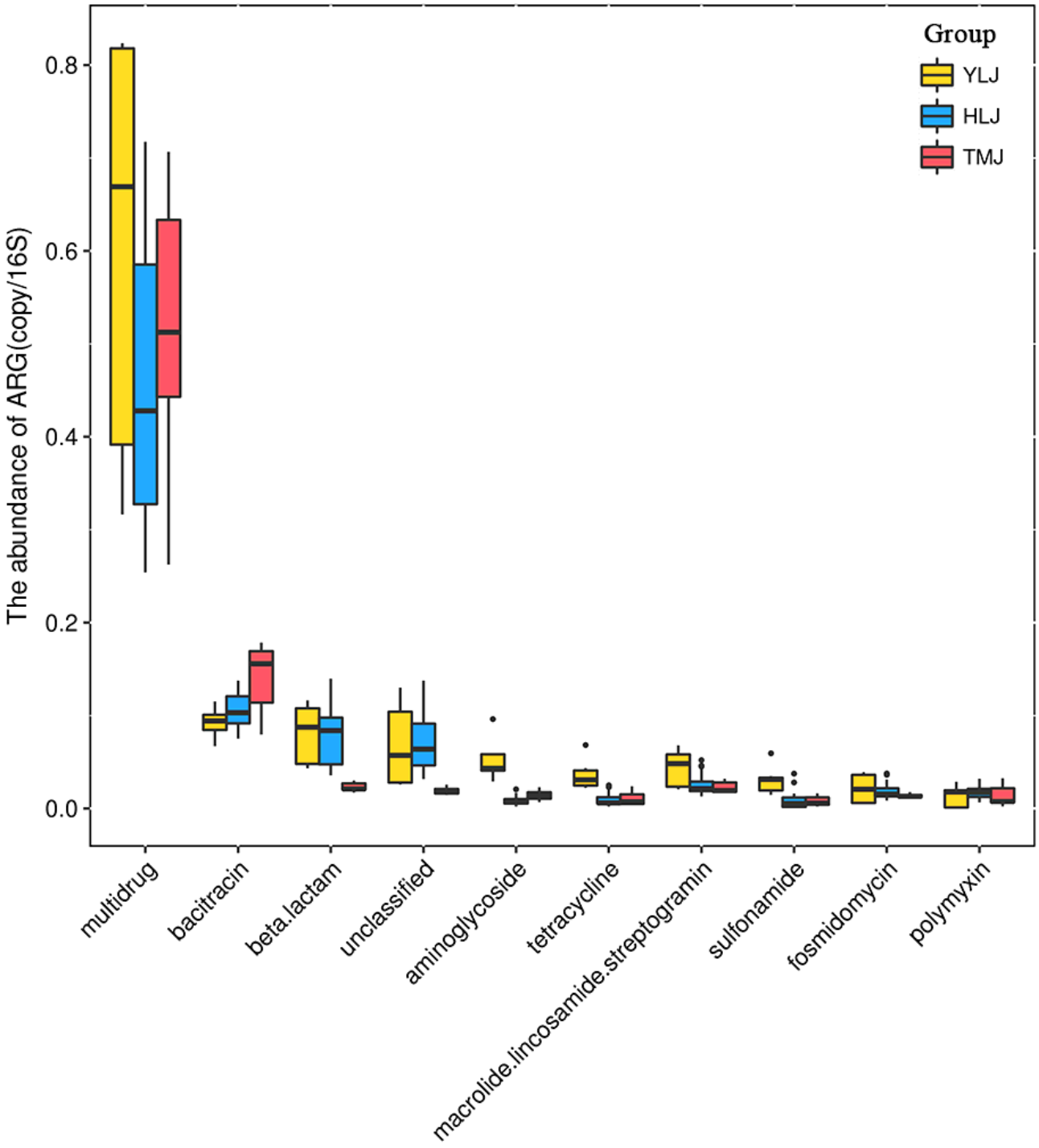

Figure 2

Abundance of the top 10 ARG types in samples from the three rivers. Zero abundances were considered in the plot.

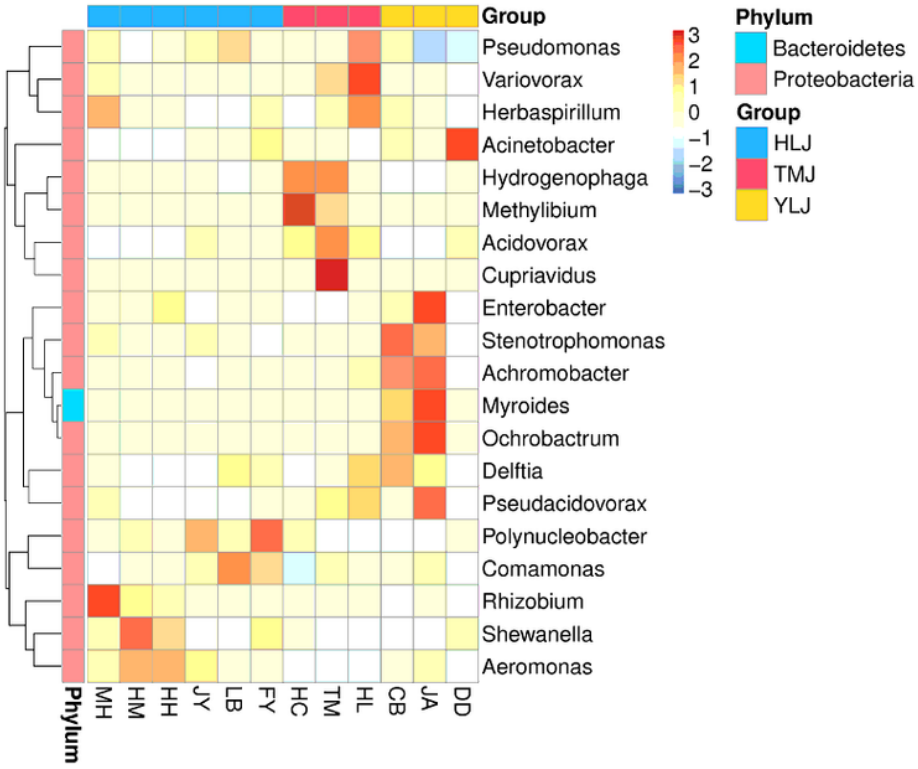

Figure 3

Bacterial community compositions at the sample sites. The top 20 bacterial genera in river water samples and their cluster patterns.

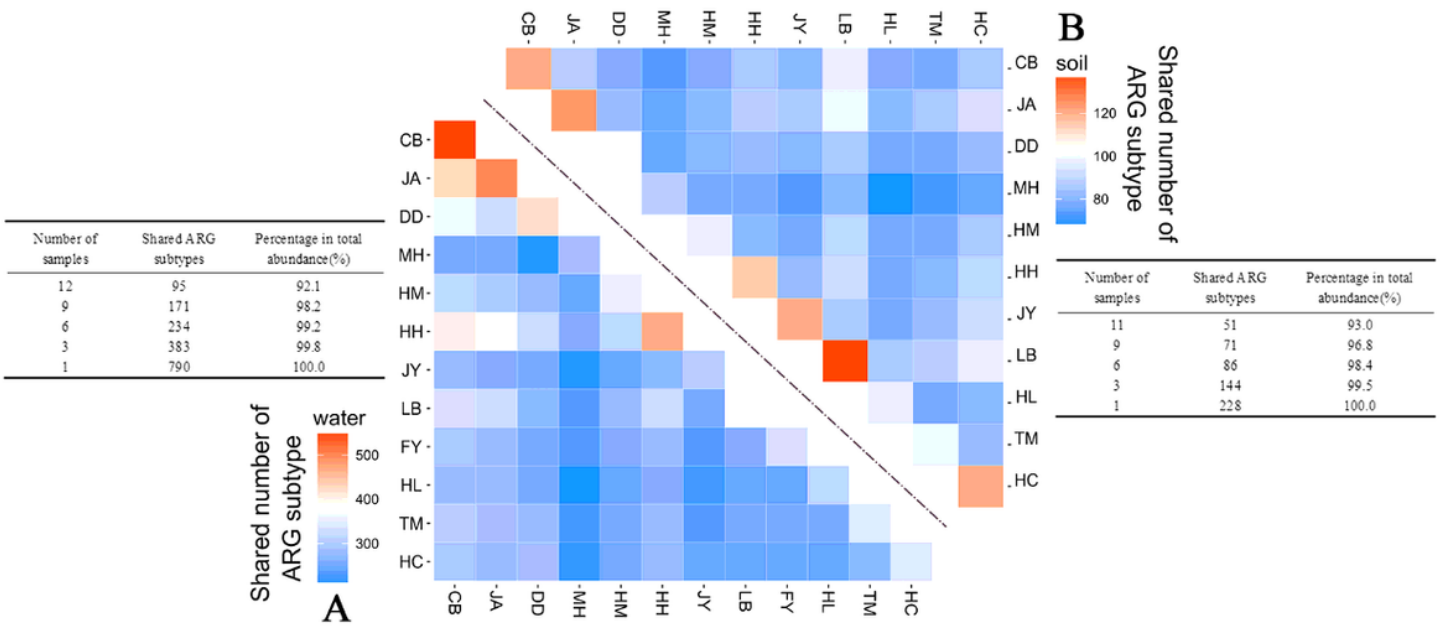

Figure 4

Shared ARG subtypes of river water samples or soil samples. A shows shared ARG subtypes in river water samples and B shows those in soil. The table shows the number of shared ARG subtypes and percentage of these shared ARG subtypes in terms of total abundance.

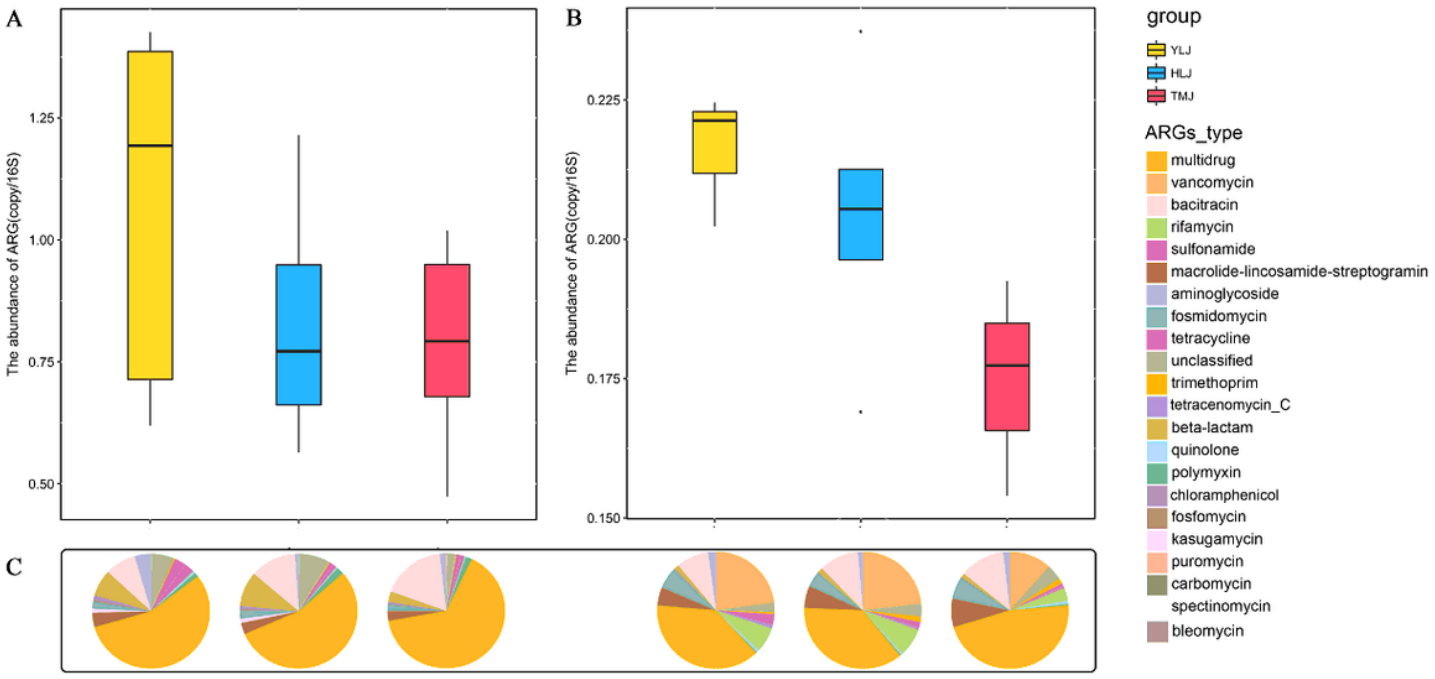

Figure 5

Abundance and composition of ARG types in river water and soil. A and B show the abundance in different groups. The pie charts below in C show top 20 ARG subtypes in different groups.

Figure 6

Composition of ARG-carrying bacteria (genus level) and the percentage of ARG types. A shows all bacteria with ARGs and B shows pathogenic bacteria with ARGs. Pie charts show the percentage of taxonomy and percentage of ARG-carrying bacteria. Bar charts show the percentages of ARG types that were determined using data from the annotated ARG-carrying bacteria.

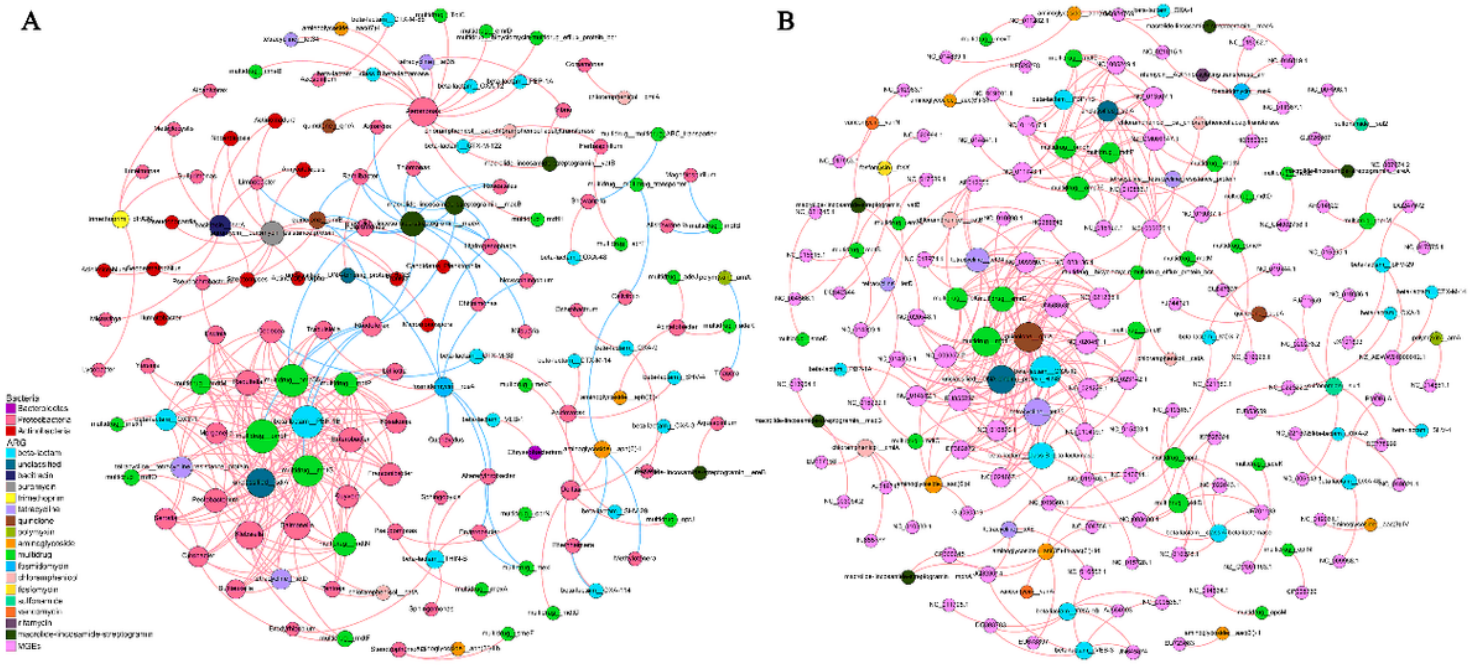

Figure 7

Correlation between ARGs and bacteria, and ARGs and MGEs based on network analysis. A shows the relationship of ARGs and bacteria; and B shows that of ARGs and MGEs. The red line indicates a positive correlation between two nodes. The blue line represents a negative correlation. The size of each node is proportional to its number of connections.

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