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Metagenomic analysis revealed the prevalence of antibiotic resistance genes in the gut and living environment of freshwater shrimp

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ABSTRACT

Antibiotic resistance disseminating from animals and their environments is a public issue that poses significant threats to human health. In the present study, the diversity and abundance of antibiotic resistance genes (ARGs) in 15 samples from the guts and related aquaculture environments (water and sediment) of shrimp were investigated. In total, 60 ARGs, 102 ARGs and 67 ARGs primarily belonging to 13, 15 and 15 different types were detected in the shrimp gut, pond water and sediment samples, respectively. Efflux pump and target modification were the predominant resistance mechanisms in all samples. It was found that *Aeromonas*, *Yersinia* and *Clostridium* XlVb were significantly correlated with the distribution of the ARGs. Besides, the relative abundance of ARGs was positively correlated with the levels of mobile genetic elements (MGEs). Moreover, variation partitioning analysis showed that MGEs, contributing to 74.46% of the resistome variation, played an important role in the affecting of the antibiotic resistome than the bacterial communities and their joint effects. Collectively, this study provides comprehensive information to better understand the ARG dissemination in aquaculture environments and to improve the ecological management of aquatic ecosystems.

1. Introduction

During the past few decades, the uncontrolled and continuous long-term use of antibiotics has contributed to the prevalence of diverse antibiotic resistance genes (ARGs) by increasing the frequency of horizontal gene transfer (HGT) and resistance gene fixation in genomes [1–3]. Currently, as emerging contaminants [4], ARGs pose a potential worldwide human health risk and are increasingly raising public concern. The World Health Organization (WHO) has identified antibiotic resistance as one of the largest threats to global health, food security and development today and has indicated that new resistance mechanisms are emerging and spreading globally, threatening the ability to treat common infectious diseases (http://www.who.int/mediacentre/factsheets/antibiotic-resistance/en/). ARGs have been identified in a wide range of environments, such as lake sediments and water [5,6], river delta and basin [7,8], livestock manure [9,10], wastewater treatment plants [11,12], drinking water [13,14] and aquaculture farms [15,16]. Most of these ARG occurrences are related to anthropogenic activities [17].

Antibiotics used in animal farming and industrial livestock production account for a large portion of the antibiotics produced overall. For example, in China, total antibiotic production was estimated to be approximately 210,000 tons annually of which applications in animal husbandry accounted for approximately 46% [18]. The intensive application of antibiotics in animal farming may promote the transfer of ARGs among bacteria, increasing the risk of spreading drug-resistant bacteria, particularly drug-resistant pathogens. Therefore, in recent years, much attention has been paid to ARGs in farming processes, which are regarded as one of the main human activities that contributes to the selection and dissemination of ARGs [19]. A total of 15 common veterinary antibiotics, 213 ARGs and 10 mobile genetic elements (MGEs) marker genes were detected in commercial composts from cattle, poultry and swine manures [20]. Furthermore, as one of the primary industries in China, aquaculture accounts for approximately 71% of the global aquaculture production based on a previous survey [21]. With the rapid development of the global aquaculture industry, the presence of antibiotics and ARGs in aquaculture environments is of increasing concern. Recently, a few studies focusing on the abundance and diversity of ARGs in aquaculture (mainly fish ponds) [22,23] found that fish ponds not only are reservoirs of ARGs (with abundances of up

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to 2.8×10^{-2} copies/16S rRNA gene copies) but also harbor a certain level of potential pathogenic bacteria [24]. Sulfonamide-resistance genes were prevalent, and their concentrations were the highest among the various ARGs, reflecting the widespread use of sulfonamides in aquaculture environments [25]. A study on the intestinal contents of fish indicated that feces from farmed fish contributed to the enrichment of ARGs in farm sediments [16]. Moreover, resistance genes against nine different types of antimicrobials and MGEs were observed in fish farming environments in Pakistan and Tanzania with no recorded history of antibiotic usage [26]. However, to our knowledge, few studies have evaluated the diversity and abundance of ARGs in shrimp farming environments. Moreover, the mechanisms by which ARGs are transferred among shrimp guts and the sediment and pond water of their aquatic environments remain unknown.

The objective of the present study was to investigate the occurrence, abundance and diversity of different types of antibiotics and ARGs as well as their relationships with the bacterial communities in the guts and living environments of shrimp. Based on high-throughput metagenomic sequencing, we examined the diversities and abundances of ARGs and MGEs in 15 samples, including water, shrimp gut and sediment samples, evaluated the similarities and differences of the ARG compositions among these different environmental samples, analyzed their bacterial community compositions, and revealed the correlations among the ARGs, bacterial communities and MGEs. The results demonstrated a high diversity of ARGs in freshwater shrimp guts and their living environments, and revealed that MGEs play an important role in affecting the antibiotic resistome; nevertheless, the vast majority of the genes from the sediment microbiome were previously uncharacterized novel ARGs. These findings provide comprehensive and accurate information to better understand ARG dissemination in aquaculture environments and to improve the ecological management of aquatic ecosystems.

2. Materials and methods

2.1. Sample collection and chemical analysis

Shrimp samples were obtained from five shrimp farms (LY, SZ, WX, XH and YZ) in Jiangsu, China, in April 2016. Information about the sampling sites and shrimp was summarized in Supplementary Table S1. Water used in the shrimp ponds was obtained from the nearby river and frequently exchanged (approximately once per week). The shrimp feed mainly contained protein and chlorella. Antibiotics were not used in the shrimp farming process. Sediment and pond water from each shrimp pond were also sampled. Twenty disease-free shrimps with nearly consistent size were collected from three different ponds at each farm and were transported to the laboratory within 4 h for dissection. A total of 3 L of water was collected from the center of each pond at a depth of 10 cm. In addition, five sediment samples were collected from each shrimp pond (one from the center and the other four from the four corners of each pond), and equal amounts of each of the five sediment samples were mixed together to form a composite sample (approximately 200 g). After dissection, the shrimp gut contents from each pond obtained on the same sample day were pooled for DNA extraction. The pond water samples were subsequently divided into two aliquots; one aliquot was used for antibiotic analysis and the detailed procedures were shown in the Supplementary Method S1. The other aliquot was filtered through a 0.45 μm pore size membrane to collect the bacteria for DNA extraction. After pretreatment, all samples (shrimp gut contents, membrane filtrates and sediments) were stored at −80 °C until DNA extraction.

Typical antibiotics, including sulfadiazine, sulfamethoxazole, sulfamerazine, sulfamethazine, sulfamethoxydiazine, methyl benzyl oxygen pyrimidine, norfloxacin, ciprofloxacin, lomefloxacin, enrofloxacin, ofloxacin, tetracycline, oxytetracycline, aureomycin, azithromycin and roxithromycin were measured using LC-MS. Because the antibiotic concentrations in the environmental water samples were relatively low, preconcentration was conducted prior to the measurement. Based on a previously published method [23], solid-phase extraction was performed using an Oasis® HLB cartridge (6 mL, 500 mg, Waters, Milford, MA, USA) packed with a divinylbenzene/*N*-vinylpyrrolidone copolymer with hydrophilic-lipophilic balance cartridges. For each sample, 2000 mL of filtered water was concentrated to 5 mL with a 10% aqueous MeOH solution as previously described [27].

Total nitrogen (TN), total phosphorus (TP) and total organic carbon (TOC) were analyzed according to standard methods. Water temperature (T), pH and dissolved oxygen (DO) were measured using portable meters. Detailed information of these water quality parameters is provided in Supplementary Table S2.

2.2. DNA extraction, PCR and bacterial 16S rRNA gene sequencing

DNA from the sediment, gut content and pond water samples was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals, CA, USA) according to the manufacturer's instructions. The concentration and quality of the extracted DNA were assessed using a Nanodrop 2000 (NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis. The 16S rRNA gene was amplified with a set of primers and the detailed information and procedure were described in the Supplementary Method S1. The purified PCR products were used for library preparation and high-throughput sequencing on a Miseq sequencer (Illumina, San Diego, CA, USA). The sequencing data were deposited into the Sequence Read Archive under accession number PRJNA381860. After sequencing, the paired-end reads were joined, and the potential chimeric sequences introduced during the PCR process were detected and removed using Mothur [28]. Subsequently, the high-quality reads were used to cluster preprocessed reads into operational taxonomic units (OTUs) at 97% similarity using the Quantitative Insights into Microbial Ecology (QIIME) pipeline [29]. The taxonomy of the representative sequence of each OTU was assigned with RDP Classifier.

2.3. High-throughput metagenomic sequencing and data analysis

To explore the diversity and abundance of the ARGs, all DNA samples were sent to the Novogene (Beijing, China) for library construction and high-throughput sequencing on a Hiseq 2000 sequencer (Illumina, San Diego, CA, USA). The generated quality-filtered reads were aligned using the basic local alignment search tool (BLASTx) with databases downloaded for ARGs (from the Antibiotic Resistance Genes Database (ARDB), http://ardb.cbcb.umd.edu/index.html), MGEs and integrons (from INTEGRALL, http://integrall.bio.ua.pt/), insertion sequences (ISs) [30] (from IS Finder, https://www-is.biotoul.fr/) and plasmids (from the NCBI RefSeq database; 2408 plasmid genome sequences). All parameters were set according to a previous study [31]. Parts per million (ppm), namely, reads annotated as ARGs in one million sequencing reads, was used to represent the relative abundances of ARGs in the samples [31]. Statistical analyses were conducted with the R software and the details were described in the Supplementary Method S2.

3. Results

3.1. Concentrations of antibiotics in shrimp ponds

As shown in Supplementary Table S3, the concentrations of 16 antibiotics, including sulfadiazine, sulfamethoxazole, sulfamerazine, sulfamethazine, sulfamethoxydiazine, methyl benzyl oxygen pyrimidine, norfloxacin, ciprofloxacin, lomefloxacin, enrofloxacin, ofloxacin, tetracycline, oxytetracycline, aureomycin, azithromycin and roxithromycin, were analyzed in this study. Generally, the concentration of each fluoroquinolone markedly varied, with significantly higher