ANTIBIOTIC RESIDUES, ANTIBIOTIC-RESISTANT GENES, AND MICROBIAL COMPOSITION IN THE MEKONG RIVER IN DRY SEASON

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SUMMARY

Antibiotic resistance (AMR) is among the top 10 threats to global health. Antibiotic-resistant pathogenic bacteria in rivers can affect human health via the food chain and daily activities. The present study aimed to investigate the AMR situation in the Mekong River in Cambodia and Vietnam. Antibiotic residues (20 types) were measured by ultra-performance liquid chromatography (UPLC), antibiotic-resistant genes (22 types) were investigated using targeted polymerase chain reaction (PCR), and microbial composition was analyzed by metagenomic sequencing. Sulfamethoxazole (SMX), tetracycline (TET), lincomycin (LCM), and oxytetracycline (OTC) were found in water samples in Vietnam and Cambodia with concentrations of *Acinetobacter*, *Aeromonas*, *Aerococcus*, *Burkholderia*, *Chromobacterium*, *Enterobacter*, *Enterococcus*, *Flavobacterium*, *Flexcibacter*, *Pseudomonas*, *Mycobacterium* and *Vibrio*. Sulfonamide resistance genes (*sul1, sul2, sul3*), macrolides resistance gene (*erm(B)*), and tetracycline resistance genes (*tetQ, tetM*) were found in 16, 11, and 5 samples, respectively. Bacterial composition analysis revealed 61 bacterial genera in which various human pathogens were detected including. These findings could be used to assess the risks of transmission and infection with antibiotic-resistant bacteria for the population in the Mekong River Basin region.

Keywords: Antibiotic-resistant genes, antibiotic residues, Mekong River, metagenomics sequencing, pathogenic bacteria.

INTRODUCTION

The emergence and dissemination of antimicrobial resistance (AMR) has turned into a major public health threat worldwide (ARC 2022). Antibiotic-resistant bacteria can be found in all known ecosystems. An in-depth understanding of the mechanisms of AMR emergence and dissemination is essential for the development of interventions and strategies to control this global threat (Kim and Cha, 2021). Globally, human activities have drastically modified environments by introducing contaminants and compounds that adversely affect ecosystems and microbial communities. The water environment is one of the most illustrative examples. Rivers and streams are central to human activities through their use for trade, transport, navigation, industries, irrigation, electricity production, as well as domestic and leisure activities (Liu *et al*., 2018). They are a major source of drinking water both for humans and animals (domestic or wild). They also receive effluents from multiple human activities and can transport all kinds of pollutants (plastic, pesticides, metals, drug residues...) over huge distances. Among these pollutants, antibiotic residues are known to contribute to the selection and amplification of AMR in humans, livestock, and the environment. Their accumulation and dispersion in water environments are a result of human and animal (mis)-use, release from industrial, hospital, or community effluents, or bad wastewater treatment practices (Grenni, 2022; Singh *et al.,* 2019; Shin *et al*., 2023). Some activities, such as the agricultural spread of manure and sewage disposal, are suspected to introduce antibiotic-resistance genes (ARGs) and resistant microorganisms into the aquatic environment, creating new interactions amongst bacterial populations. These places therefore turn into incubators gathering bacteria from different origins, antibiotics, disinfectants, and metal pollutants, favoring intra- and inter-specific horizontal gene transfers (HGT) between bacteria and the emergence and dissemination of antibiotic resistance. Aquatic environments therefore represent a unique setting for the acquisition and spread of ARGs, as well as for the proliferation of resistant bacteria.

The Mekong River flowing from its source in the Tibetan plateau through China, Myanmar, Thailand, Laos, Cambodia, and Vietnam, is an excellent illustration of the interactions that can take place between rivers and human communities. Antibiotic-resistant bacteria infections in Vietnam and Cambodia are increasing for a variety of reasons, including poor medication quality, insufficient AMR surveillance, low community awareness, insufficient regulation, excessive agricultural use, economic drivers, and overuse of antimicrobials, of which antibiotics is a subset (McKinn *et al*., 2021). Nevertheless, the role and impact of the Mekong River on the emergence and dissemination of antibiotic-resistant bacteria were poorly investigated in Vietnam and Cambodia. The present study investigated the contamination of antibiotics and antibiotic-resistant genes in the Mekong River

in Vietnam and Cambodia. Our findings may be useful in supporting governments in developing different strategies for controlling AMR in the Mekong Delta Basin region.

MATERIALS AND METHODS

Water collection

A total of 27 water samples were collected along the Mekong River in Cambodia (9 stations) and in Vietnam (18 stations). For each location, 10 liters (L) of water were taken at 5 centimeters (cm) below the top of the surface water and then aliquoted into 500 ml sterilized bottles. These bottles were immediately stored in the ice box and transported to the laboratory for further analysis.

Quantification of Antibiotic residues

Ultra-performance Liquid Chromatography (UPLC) coupled with a tandem mass spectrometer (Waters, Xevo-TQD, USA) was used to quantify 20 target compounds including ofloxacin, ofloxacin D3, ciprofloxacin, norfloxacin, trimethoprim, sulfamethoxazole, 13C6-sulfamethoxazole, carbamazepine, ampicillin, amoxicillin, amoxicillin 13C6, tetracycline, azithromycin, cefotaxime, lincomycin, roxithromycin, clarithromycin, erythromycin, oxytetracycline, doxycycline. Target analytes were separated on a C18 column (BEH, C18, 50 x 2.1 mm, 1.7 µm particle size). Standard solution concentrations (10-500 ppb) were prepared for the calibration curves. Each calibration curve was linear with a coefficient of determination (R2) >0.995. For this test, two samples with concentrations of 10 µg/L and 500 µg/L were prepared by spiking target compounds into river water. Relative recoveries were calculated using the following equation:

$$\% \text{ Relative recovery} = (CS - CM)/(C0) \times 100 \quad (1)$$

where CS and CM are the concentrations of the analyte determined for the spiked sample and the un-spiked matrix, respectively, and C0 is the predetermined concentration of the spiked sample. The MDL (Method detection limit) was determined to be 3.143 times (the Student t value for six degrees of freedom at a 99% confidence level) the standard deviation of three replicate samples within the dynamic range (US EPA, U.S. Code of Federal Regulations).

Environmental DNA extraction and Detection of antibiotic-resistant genes

For each station, 1 litter of water sample was filled by 0.2 ul filter membrane to collect bacterial biomass. The membrane was then cut into small pieces and used for environmental DNA (eDNA) extraction using a DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the manufacturer's guidelines. The quantity and purity of eDNA were measured using Nanodrop 2000 (Thermofisher, USA).

22 AMR genes associated with resistance to 13 antibiotic resistance groups were screened by targeted PCR using specific primers. 13 antibiotic resistance groups included: Sulfonamide (*sul1, sul2, sul3*), macrolides (*erm(B)*), tetracycline (*TetQ, TetM*), trimethoprim (*dfrA*), colistin (*mcr-1*), Quinolone (*QnrA, QnrS, QepA*), methicillin (*MecA*), Carbapenem (*OXA-48, KPC, NDM*), Integrons (*int*), vancomycin (*vanA*), beta-lactam (*CMY*). 1 µL DNA template was added to the 19 µL master mix consisting of 15 µL sterile water, 2 µL 10X buffer, 0.4 µL dNTPs, 0.8 µL Taq polymerase, 0.4 µL of each primer. Cycling parameters were 95°C for 2 min followed by 35 cycles of 95°C for 30s, 52-60°C (Depending on annealing temperature) for 15 s, 72°C for 5 min, and finally 72°C for 10 min. The PCR products were run through a 1% agarose gel stained with Red Safe (Intron, Korea). Non-template controls (NTCs) were used for all experiments.

Metagenomics sequencing analysis

To identify the composition and number of prokaryotic microorganisms, 16S rDNA amplicon sequencing involves building libraries using specific primers to amplify the variable region V3-V4 of bacterial 16S rDNA. The DNA extraction from the samples amplified the two variable regions of 16S rDNA (V3 and V4) and accurately identified various species including archaea. Data were analyzed using MG-RAST with default parameters to determine the microbial makeup of the individual sampling sites. The MG-RAST automated analysis process makes use of the M5nr (MD5-based non-redundant protein database) which integrates many sequence databases (EBI, GO, JGI, KEGG, NCBI) for annotation. The process separates after uploading samples with 16S ribosomal amplicons. The WGS workflow is divided into several stages, beginning with the removal of low-quality reads, and progressing to dereplication, gene calling, annotation, and the creation of functional abundance profiles. On rRNA samples, RNA detection, clustering, and identification are conducted, and taxonomic abundance profiles are generated as a result (Keegan et al., 2016).

RESULTS AND DISCUSSION

Quantification of antibiotic residues in the Mekong River

Five antibiotic residues including SMX, TET, LCM, CTX, and OTC were detected in the Mekong River in Cambodia and Vietnam (Figure 1). Specifically, CTX was found in all 9 stations in Cambodia with high

concentration (from 35.1 µg/L to 60.3 µg/L), while it was detected in only 6 stations in Vietnam (from 35.2 µg/L to 48.5 µg/L). The TET was mainly found in Vietnam (n=10, 23 – 80 µg/L), while CTX was detected in all samples from Cambodia (38 – 60 µg/L). The LCM was found in both Vietnam (n=4) and Cambodia (n=4) with concentrations of greater than 120 µg/L. The OTC was detected in 14 samples with the concentration ranging from 28.9 µg/L to 147.4 µg/L. The MDLs ranged from 0.2–11.5 ng/L for the range of antibiotics investigated. Spiked MDL samples were produced at concentrations 2–5 times that of the predicted detection limit of each drug. The MDLs determined in our investigation are similar to those determined by the US EPA Method. Overall, antibiotic residues were accumulated in the Mekong River Basin in Vietnam. The largest global study found antibiotics in 65% of 711 sites in rivers from 72 countries. Of note, the concentrations of antibiotics exceeded safe levels in 111 sites, particularly some sites more than 300 times over the safe limit. Our study underlines the high level of antibiotic contamination in the Mekong River. Thus, the impact of antibiotic contamination on the emergence and transmission of antibiotic-resistant bacteria in the River needs to be investigated.

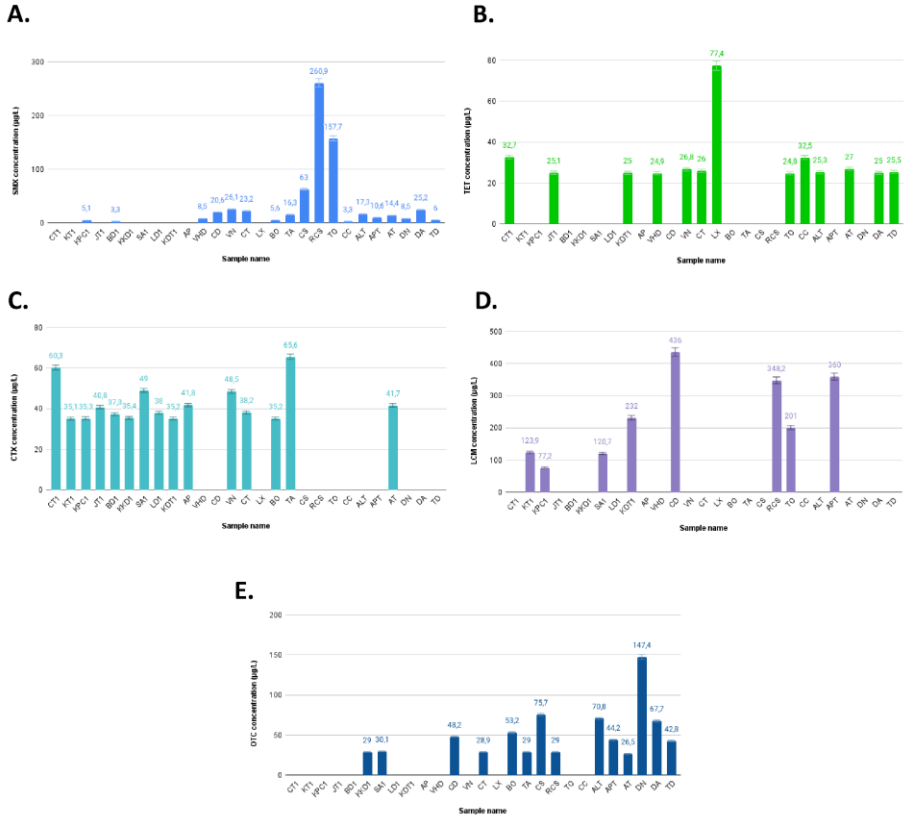

Figure 1. Antibiotic residues detected in the Mekong River. SMX (A), TET (B), CTX (C), LCM (D), and OTC (E)

Detection of antibiotic-resistant genes

Six ARGs were identified in the Mekong River, including *sul1, sul2, sul3, erm(B), tetQ,* and *tetM*. Specifically, *Sul1* was detected in 10 samples of Vietnam but not in any samples of Cambodia. *Sul2* was detected in three samples of Cambodia, while *Sul3* was found in three samples of Vietnam. The *erm(B)* was found in 11 of the 27 samples. Finally, genes responsible for tetracycline resistance *TetM* and *tetQ* were detected in one sample of Cambodia and four samples of Vietnam, respectively. A previous study reported the presence of *sul1* and *sul2* along with the *blaCTX* in the Mekong River (Nakayama *et al*., 2017). Our findings are in agreement with global studies where genes resistant to sulfonamide (*sul*1, *sul*2, and *sul*3), and tetracycline (*tet*A, *tet*B, *tet*C, *tet*M, *tet*O, *tet*X, *tet*W) were the most commonly detected in almost river systems (Cacace *et al*., 2019; Lee *et al*., 2020; Raza *et al*., 2022; Zheng *et al*., 2022).

Distribution of potential pathogenic bacteria in the Mekong River

Analysis of bacterial composition revealed 61 bacterial genera in the studied samples, in which *Exiguobacterium, Streptomyces, Synechococcus, Acinetobacter,* and *Pseudomonas* were the most dominant (Figure 2). Notably, 24 bacterial genera were only found in Vietnam, while 16 bacteria genera were detected in Cambodia. Bacterial composition was different by sampling regions and by countries. Samples from the same country had more similar bacterial composition than samples belonging to different countries. Alpha diversity analysis demonstrated that bacterial richness and diversity were significantly higher in Vietnam than in Cambodia (Figure 3).

Furthermore, potential pathogenic bacteria associated with human and animal infections were found including *Aeromonas, Aerococcus, Mycobacterium, Vibrio, Flavobacterium, Flexcibacter, Burkholderia, Enterobacter, Pseudomonas, Acinetobacter, Chromobacterium,* and *Enterococcus.* In agreement with previous studies, *Enterobacteriaceae*, *Moraxellaceae*, *Pseudomonadaceae*, and *Vibrionaceae*, dominant in aquatic environments clinically significant pathogenic bacteria, and, are the major hosts of antibiotic-resistant genes (Kim and Cha, 2021; Shin *et al*., 2023). Notably, antibiotic-resistant genes are highly exchanged and disseminated within bacterial strains and between species of these pathogens, and consequently enhance the bloom of antibiotic-resistant genes-carrying species. Multidrug-resistant *A. baumannii*, *E. coli*, and *P. aeruginosa* strains belonging to the priority pathogen list of WHO are also detected in aquatic environments. Furthermore, antibiotic residues, metals, and various wastes foster the spread of antibiotic-resistant genes in environmental bacteria with the help of mobile genetic elements through horizontal gene transfer events (Kim and Cha, 2021; Shin *et al*., 2023). Aquatic environments thus represent a unique setting for the acquisition and spread of antibiotic-resistant genes, as well as for the proliferation of antibiotic-resistant bacteria (Lee *et al*., 2020). Therefore, the presence of antibiotics, pathogenic bacteria, and antibiotic-resistant genes in the Mekong River underlines that the river is an important reservoir for the emergence and spread of antibiotic-resistant bacteria in the population. These data could be used by the stakeholders as scientific evidence for actions and regulation implementation or modifications to better control the emergence of AMR in this region.

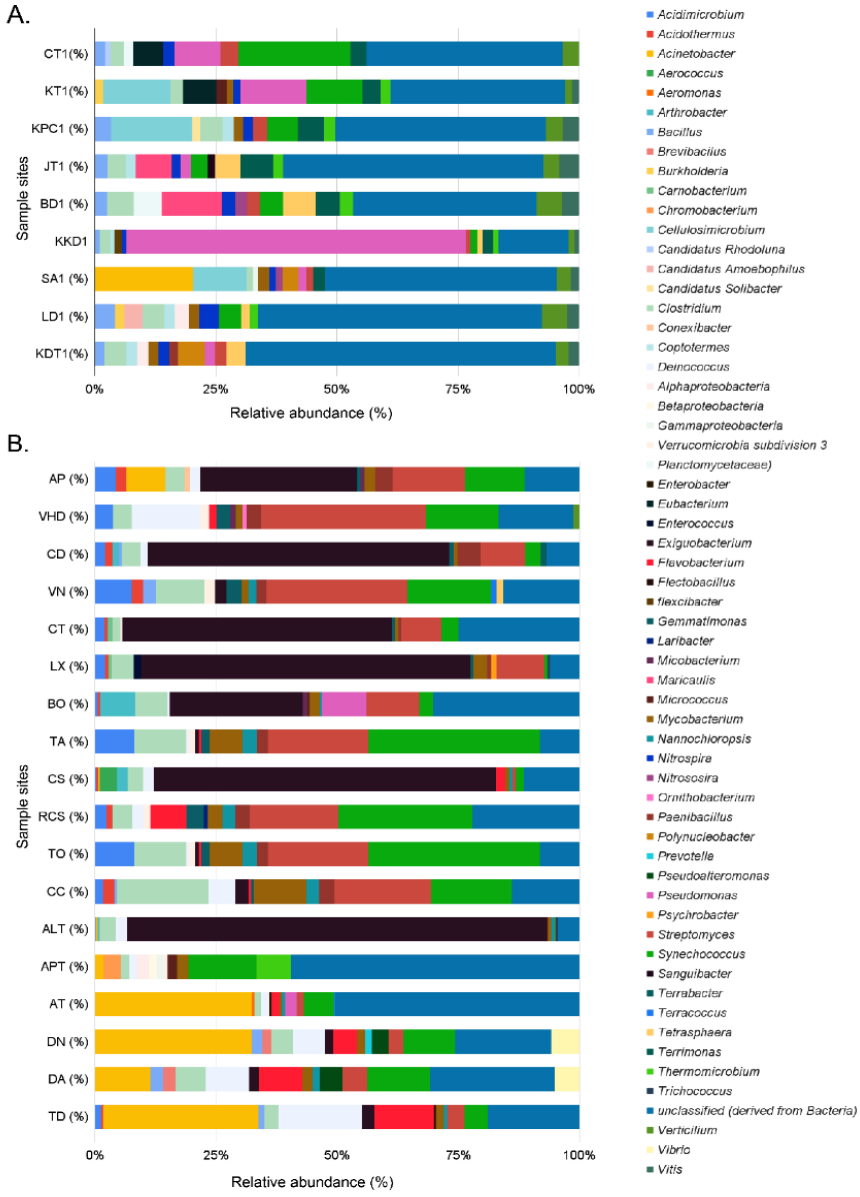

Figure *2*. Composition and distribution of bacteria in the Mekong River in Cambodia (A) and Vietnam (B)

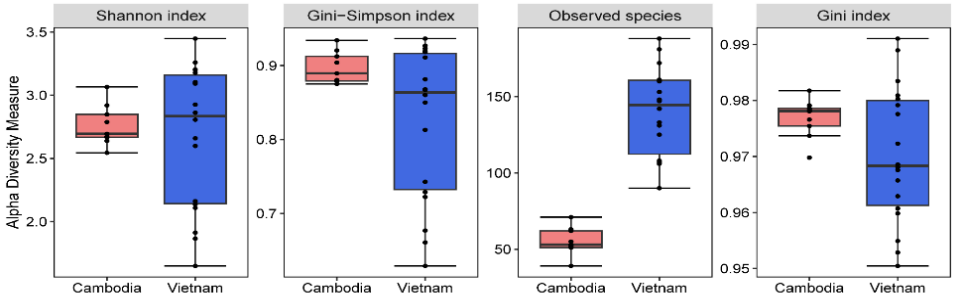

Figure 3. Bacterial diversity in surface water in the Mekong River

CONCLUSION

The Mekong River is contaminated with antibiotic residues, antibiotic-resistant genes, and pathogenic bacteria. The river can be a potential reservoir for the emergence of antibiotic-resistant pathogenic bacteria which are risks to human and animal health and ecosystems.

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KHÁNG SINH, GENE KHÁNG THUỐC VÀ THÀNH PHẦN VI KHUẨN Ở SÔNG MEKONG TRONG MÙA KHÔ

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TÓM TẮT

Kháng kháng sinh nằm trong 10 mối đe doạ nghiêm trọng nhất đối với sức khoẻ toàn cầu. Vi khuẩn kháng kháng sinh trong sông có thể lây nhiễm tới người thông qua chuỗi thức ăn và các hoạt động hằng ngày. Nghiên cứu này đánh giá thực trạng kháng kháng sinh ở Sông Mekong lưu vực Campuchia và Việt Nam. Dư lượng kháng sinh (20 loại) được xác định bằng hệ thống sắc ký lỏng hiệu năng cao (UPLC), gene kháng thuốc (22 loại) được xác định bằng PCR gene đích, thành phần vi khuẩn được phân tích từ dữ liệu giải trình tự metagenomics. Các kháng sinh gồm Sulfamethoxazole, tetracycline, lincomycin, và oxytetracycline có sự tồn dư trong nước khá cao với nồng độ từ 20 – 450 µg/L. Các gene kháng Sulfonamide (*sul1, sul2, sul3*), macrolides (*erm(B)*) và tetracycline (*tetQ, tetM*) được phát hiện lần lượt ở 16, 11 và 5 mẫu nghiên cứu. Phân tích thành phần vi khuẩn xác định được 61 chi vi khuẩn, trong đó vi khuẩn là mầm bệnh của người như *Acinetobacter, Aeromonas, Aerococcus, Burkholderia, Chromobacterium, Enterobacter, Enterococcus, Flavobacterium, Flexcibacter, Pseudomonas, Mycobacterium* và *Vibrio*. Những phát hiện này có thể được sử dụng để đánh giá nguy cơ lây truyền và nhiễm vi khuẩn kháng thuốc trong cộng đồng cư dân ở lưu vực sông Mekong.

Từ khóa: Dư lượng kháng sinh, giải trình tự metagenomics, gene kháng thuốc, sông Mekong, vi khuẩn gây bệnh.

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