Preplanned Studies

Occurrence and Removal of Antibiotic Resistance in Nationwide Hospital Wastewater Deciphered by Metagenomics Approach — China, 2018–2022

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Summary

What is already known about this topic?

Hospital wastewater represents a significant conduit for the dissemination of antibiotic resistance genes (ARGs), including those that are of clinical importance. Yet, the variation in antibiotic resistance on a national level, which can be influenced by regional differences, has not been thoroughly elucidated.

What is added by this report?

Uniform methodologies were employed to analyze data from various provincial-level administrative divisions in China, aiming to investigate the patterns of antibiotic resistance across these regions. This analysis illuminated the location preferences of clinically ARGs on plasmids. Additionally, our study uncovered regional variations in the presumed bacterial hosts of ARGs at the genus level.

What are the implications for public health practice?

This investigation has shed light on the nationwide prevalence of ARGs in hospital wastewater in China, uncovering the significant ecological and public health risks associated with the disposal of such effluents.

Antibiotic resistance has emerged as a significant threat to global public health. The excessive use of antibiotics is known to hasten the spread of antibiotic resistance. The selective pressure exerted by antibiotics is exceptionally high in acute care hospitals, where an estimated 20%–30% of inpatients in Europe are administered antibiotics during their hospitalization (*1*). Hospital wastewater typically contains higher concentrations of antibiotics and antimicrobials than municipal wastewater (*2–4*), making it a critical reservoir for antibiotic resistant bacteria (ARB) and a conduit for the proliferation of these resistant strains (*2,5*). The occurrence of pathogens resistant to "last-resort" antibiotics in hospital settings, combined with the nosocomial transmission of such resistant mechanisms, represents a profound risk to patient

health and the surrounding environment (*4*).

In this study, 78 metagenomic datasets of hospital wastewater from six Chinese provincial-level administrative divisions (PLADs) were either obtained from the National Center for Biotechnology Information (NCBI) database or generated through direct collection and sequencing. Employing standardized methods, the research set out to analyze data from diverse regions within China to explore regional variations in antibiotic resistance. The investigation revealed that numerous clinically ARGs are carried on plasmids. Notably, the study identified regional differences in the potential bacterial hosts of ARGs at the genus level. Additionally, it was found that the existing hospital wastewater treatment systems are insufficient in effectively removing antibiotic resistance, further emphasizing the substantial ecological and health hazards associated with hospital wastewater discharge.

Metagenomic datasets representing hospital wastewater in China, spanning the years 2018 to 2022, were retrieved from the NCBI database. Additionally, we collected both influent and disinfected effluent samples from a general hospital in Shenzhen City, Guangdong Province, China. The samples underwent metagenomic sequencing on the Illumina NovaSeq 6000 platform, facilitated by Novogene (Tianjin, China), utilizing a paired-end 150 bp sequencing approach. Comprehensive details of the public datasets are presented in Supplementary Table S1 (available in https://weekly.chinacdc.cn/). To quantify the abundance of ARGs in each sample, we employed the standardized ARGs-OAP pipeline in conjunction with the SARG database (Version 3.2, https://smile.hku.hk/ARGs/Indexing), conforming to the suggested parameters and thresholds (*6–7*).

Quality assessment of the raw paired-end reads was conducted using Fastp (Version 0.22.0, https://github.com/OpenGene/fastp), ensuring that only reads of quality score above 20 were retained for subsequent analysis. These high-quality reads from each sample

were individually assembled into contigs with MegaHit (Version 1.2.9, https://github.com/voutcn/megahit). Predictions of open reading frames (ORFs) were executed using Prodigal (Version 2.6.3, http://prodigal.ornl.gov/). We utilized Platon in accuracy mode to differentiate putative plasmid-derived contigs from chromosomal contigs, ensuring meticulous discrimination. Subsequent alignment of ORFs on these assembled sequences against the SARG database was performed (8). For contigs presumed to be of chromosomal origin as determined by Platon, taxonomic classification was executed using Kaiju (Version 1.9.2, https://bioinformatics-centre.github.io/kaiju/). The relative abundance of ARG-carrying contigs (ACCs) was calculated in accordance with methodologies previously described in the literature (9).

This study identified a total of 1,899 ARG subtypes across 28 different ARG types within the wastewater samples analyzed (Figure 1A). Principal Coordinate Analysis (PCoA) based on the prevalence of ARG subtypes demonstrated distinct clustering of samples from Hebei, Guangdong, and Hunan provinces (Figure 1B). Conversely, in Sichuan Province, samples from the same hospital exhibited more similar ARG profiles. A significant positive correlation was observed between the aggregate abundance of ARGs and the presence of ARG-harboring plasmids (Figure 2B). A diversity of ARGs localized on plasmids was observed, with several subtypes such as aadA, qacEΔ1, cmlA5, sul1, catB3, ereA, OXA-10, and OXA-1 frequently co-occurring. Notably, plasmids routinely carried numerous clinically significant ARGs, including CTX-M-3, KPC-2, NDM-6, and the vanM gene cluster (Figure 2C). At the phylum level, Pseudomonadota was consistently the most prevalent ARG host across all PLADs (Figure 3A). However, at the genus level, variations in ARG genomic landscape were identified across different regions (Figure 3B). Pathogenic genera such as *Escherichia*, *Acinetobacter*, *Aeromonas*, *Pseudomonas*, *Klebsiella*, and *Enterococcus* were associated with a higher diversity of ARGs when compared to non-pathogenic genera.

DISCUSSION

In this study, the prevalence of antibiotic resistance in hospital wastewater across a national scope was evaluated using standardized methodologies that employed both read-based and assembly-based metagenomic analysis. This examination uncovered distinctions in the profiles of ARGs, with geographical location and hospital type significantly impacting the distribution of these ARGs (Supplementary Figure S1, available in https://weekly.chinacdc.cn/). Although there was variance in the overall abundance of ARGs across different regions, the predominant categories of ARGs, including those conveying resistance to aminoglycosides, beta-lactams, macrolide-lincosamide-streptogramin (MLS), sulfonamides, and tetracyclines, remained consistent across PLADs (10). It is likely that the observed geographical influence can be attributed to regional differences in antibiotic prescription trends. Similarly, the type of hospital appeared to directly affect the variation in antibiotic use (3).

Mobile genetic elements (MGEs) are well-documented vehicles for the transference of ARGs, facilitating their propagation (11). This study hones in on plasmids, a critical type of MGE capable of transferring ARGs across diverse phylogenetic boundaries. Our analysis identified a significant positive correlation between the prevalence of ARGs and the presence of plasmids harboring these genes, underscoring plasmids' role in the mobilization of ARGs. The distribution of ARGs on plasmids was particularly telling, as numerous ARGs with clinical significance were found to be plasmid-borne, highlighting the considerable risk of their transference among bacterial species. Corroborating previous research, our study also detected the vanM gene cluster (4), which imparts resistance to vancomycin, on plasmids. Initially discovered in an Enterococcus faecium isolate from Shanghai Municipality, China, in 2006, vanM has been prevalent in the region since then (12–13). The detection of vanM in hospital wastewater on a national scale indicates widespread dissemination and points to plasmids as a likely contributor to this trend. Our findings demonstrate the pervasive presence of clinically significant ARGs on plasmids, suggesting that plasmids play a pivotal role in the dissemination of ARGs.

Upon further investigation into the ARG genetic context, we discovered that Pseudomonadota consistently emerged as the principal ARG carrier at the phylum level, regardless of the geographic PLADs. At the genus level, however, we observed pronounced regional variations in the ARG genetic context. Notably, the genus *thauera* exhibited a significantly higher ARG carrier abundance in Hebei Province compared to other PLADs, whereas the genus *Acinetobacter* was more abundantly identified as an ARG carrier in Gansu Province ($P<0.05$). Additionally,
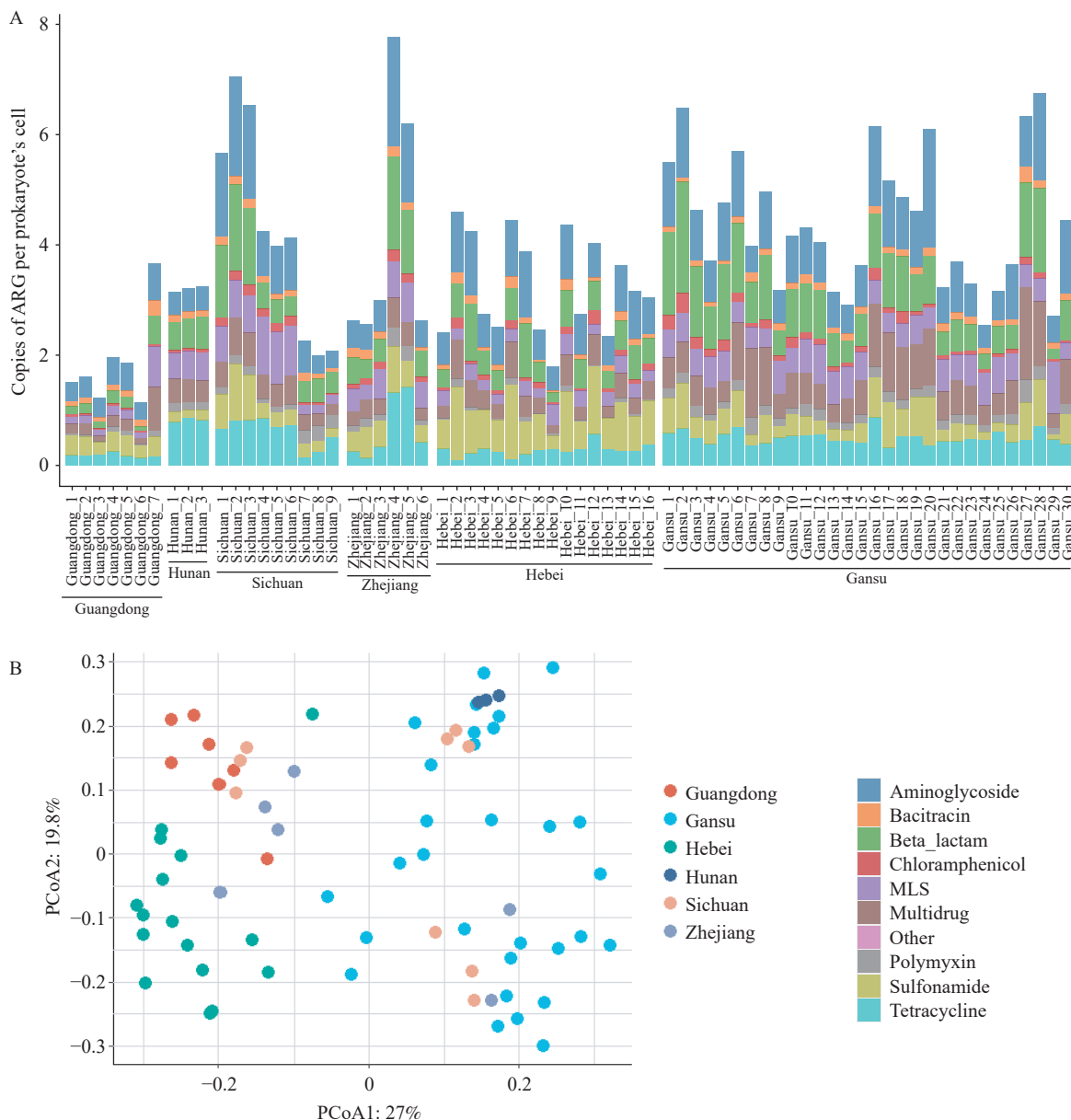
FIGURE 1. The occurrence of ARGs in untreated hospital wastewater. (A) The abundance of ARGs in untreated hospital wastewater sampling from different provinces. (B) PCoA analysis based on Bray-Curtis distance of ARG subtypes' abundance.
Note: Samples including Sichuan_1, Sichuan_2, and Sichuan_3 were taken from a general hospital while Sichuan_4, Sichuan_5, Sichuan_6 were taken from a hospital of traditional Chinese medicines, and Sichuan_7, Sichuan_8, Sichuan_9 were taken from a hospital of stomatology. Samples including Zhejiang_1, Zhejiang_2 and Zhejiang_3 were taken from an eye hospital and Zhejiang_4, Zhejiang_5, Zhejiang_6 were taken from a general hospital.
Abbreviation: MLS=macrolide-lincosamide-streptogramin.

our findings indicated that pathogenic genera harbored a greater diversity of ARGs compared to their non-pathogenic counterparts (Figure 3C). Complementing these observations, investigation of metagenomic draft genomes from hospital wastewater environments revealed that approximately 60% of the identified potential pathogens (*14*). Among the *E.coli* resistant to erythromycin, ampicillin, or trimethoprim/ sulfamethoxazole isolated from the same fecal specimen of children, pathogenic isolates were found to be approximately as twice as non-pathogenic isolates (*15*). This result might be related to the clinical usage of antibiotics against pathogens, as previously confirmed that hospital-specific antibiotic usage was generally associated with increased hospital antibiotic susceptibility (*16*).
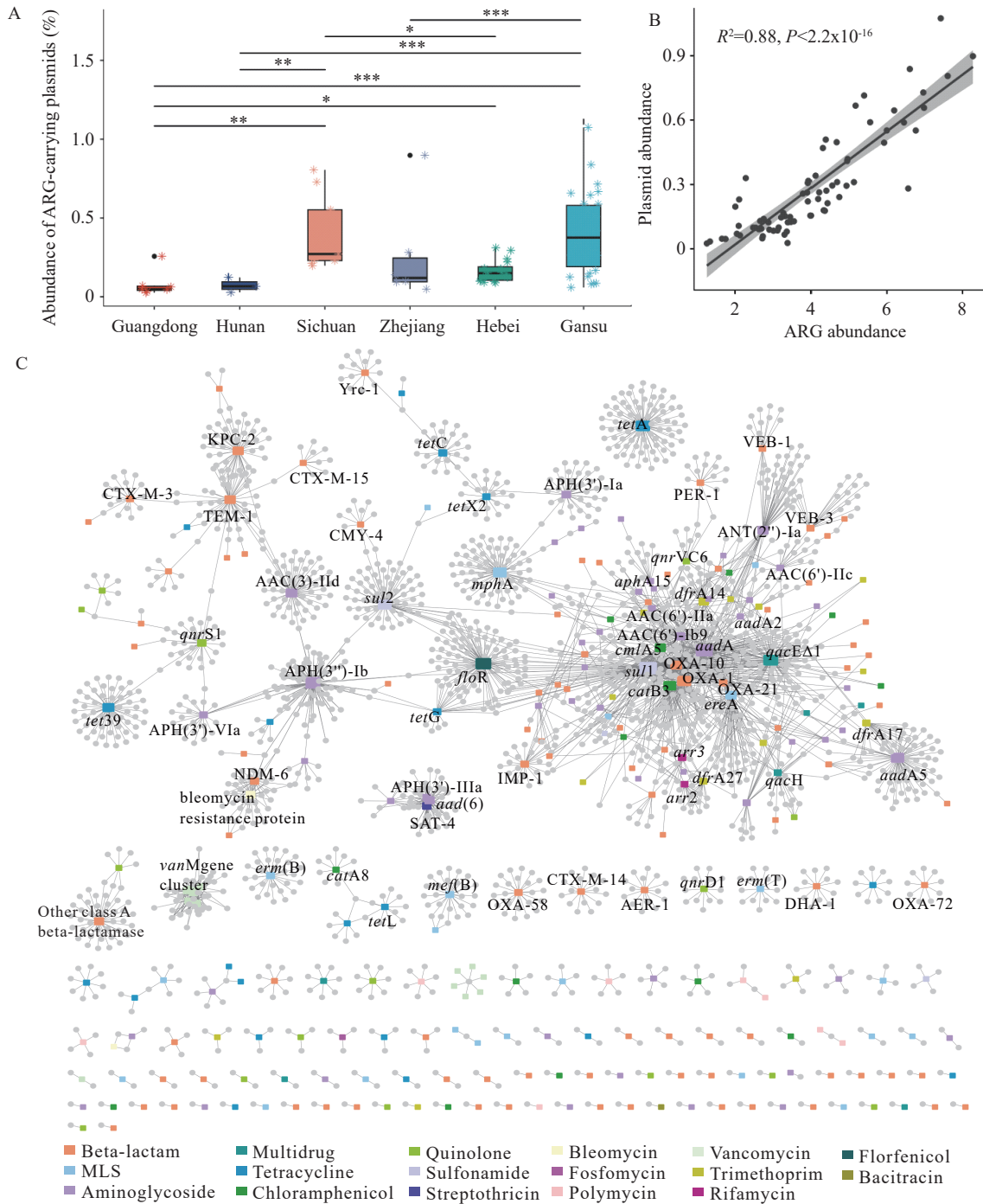
FIGURE 2. The profile of ARGs located on plasmids. (A) The abundance of ARG-carrying plasmids in different provinces. (B) The Pearson correlation between the total abundance of ARGs and the abundance of ARG-carrying plasmids. (C) The ARG-carrying pattern on the putative plasmid contigs.

Note: The ARGs with more than 10 connections to the plasmid contigs were labeled in the figure.

Abbreviation: MLS=macrolide-lincosamide-streptogramin; ATGs=antibiotic resistance genes.

* $P<0.05$;

** $P<0.01$;

*** $P<0.001$.

Wastewater treatment processes are not always effective at eradicating ARGs. Notably, while ARGs were found to be significantly reduced in the wastewater from a general hospital in Zhejiang Province, the total abundance of ARGs in the treated wastewater of eye hospitals actually increased, rather
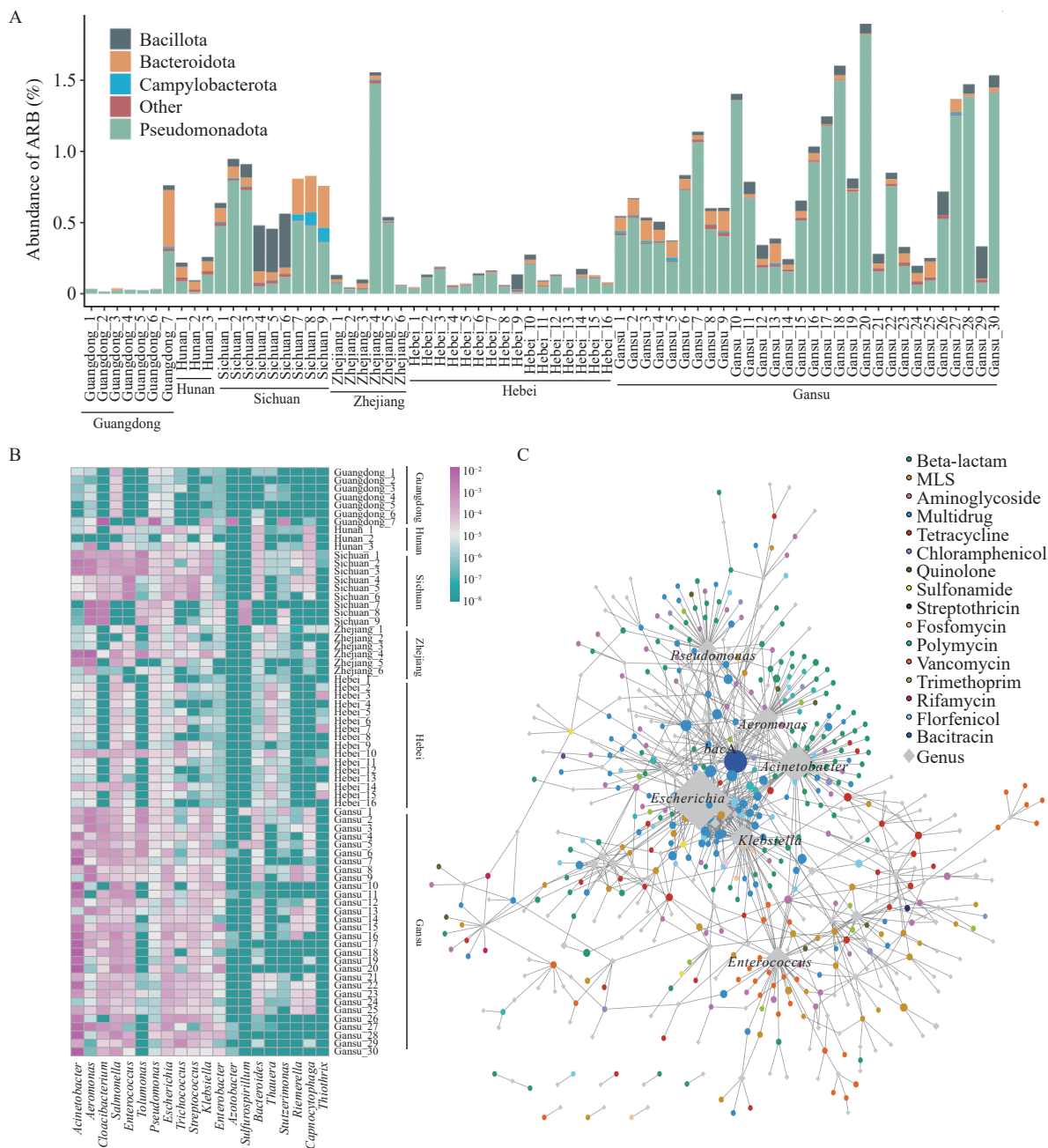
FIGURE 3. The genetic context of ARGs. (A) The genetic context of ARGs at phylum level. (B) The genetic context of ARGs at genus level. (C) The ARG-carrying patterns of different genera.
Abbreviation: MLS=macrolide-lincosamide-streptogramin; ATGs=antibiotic resistance genes.

than decreased (Supplementary Figure S2, available in https://weekly.chinacdc.cn/). This suggests that the treatment methods implemented failed to effectively eradicate bacteria harboring multiple ARGs. Prior research has demonstrated variable responses of ARGs to treatment processes, with certain genes such as *erm*B and *tet*W showing reductions while others, including bla$_{TEM}$, *sul*1, and *qnr*S, have been found to increase post-treatment (*2,17*). Zhu et al. conducted an investigation into antibiotic resistance in three hospital

wastewater treatment systems and reported that chlorination, typically the final treatment stage before wastewater discharge, was largely ineffective at significantly reducing most ARGs (*14*). However, compared to relative abundance, absolute quantification of ARGs is more significant for evaluating the environmental implications of ARGs and the effectiveness of treatment technologies. Further investigation based on the absolute abundance of ARGs is desired to explore the treatment efficiency for

reducing antibiotic resistance. Furthermore, analysis of the genetic context of ARGs revealed an increased relative abundance of pathogenic genera in treated water (Supplementary Figure S2B). Pathogenic antibiotic-resistance bacteria (ARB) such as *Aeromonas*, *Pseudomonas*, *Salmonella*, and *Klebsiella* exhibited higher prevalence in treated wastewaters (Supplementary Figure S2B). There is mounting evidence suggesting that wastewater treatment plants may actually foster an environment that supports the enrichment and proliferation of ARB and that these facilities are not adequately removing multidrug-resistant pathogens (*14*,*18*). Collectively, these findings suggest that treated wastewater could have an elevated presence of pathogenic genera that carry multiple ARGs, posing a potential risk to public health.

This investigation provides valuable insights into antibiotic resistance within hospital wastewater across China, illuminating the considerable impact of geographical location and hospital categorization on the distribution of ARGs. In addition, this study reveals a preference for certain clinically significant ARGs to be located on plasmids, as well as regional variations in the genetic context of ARGs at the genus level. The findings also demonstrate that existing wastewater treatment facilities in hospitals are inadequate in effectively eliminating antibiotic resistance. This research highlights the urgent need for enhanced management and treatment of hospital wastewater to reduce the potential spread of antibiotic resistance emanating from healthcare facilities.

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SUPPLEMENTARY MATERIAL
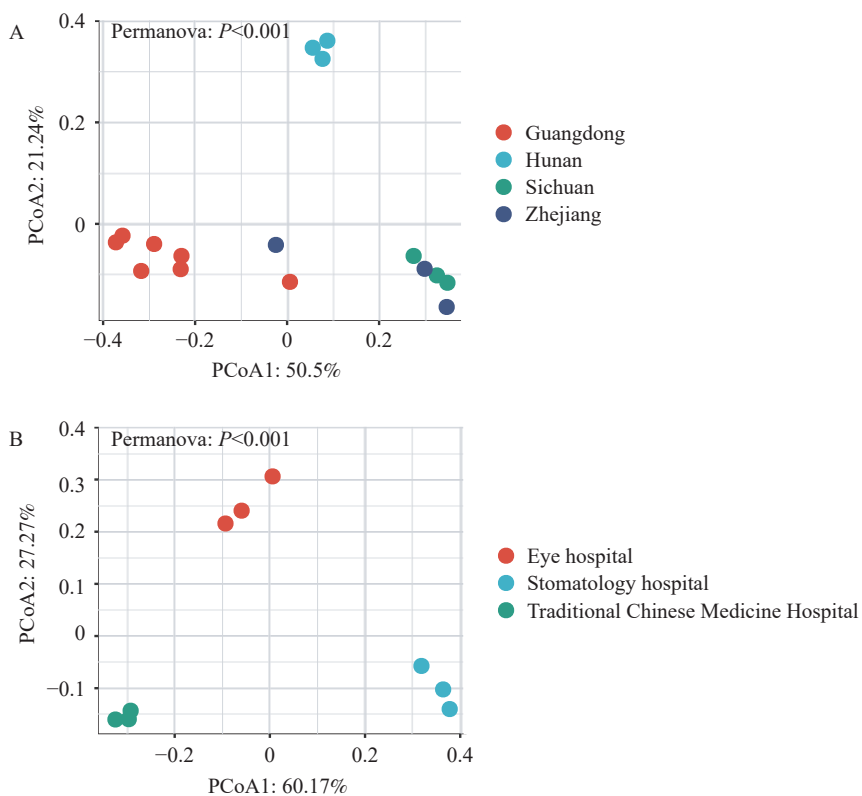
SUPPLEMENTARY FIGURE S1. The influence of geographical position and types of hospitals on ARG profiles based on PCoA analysis. (A) Analysis based on Bray-Curtis distance of ARG subtypes' abundance from different general hospitals. (B) Analysis based on Bray-Curtis distance of ARG subtypes' abundance from different types of hospitals.
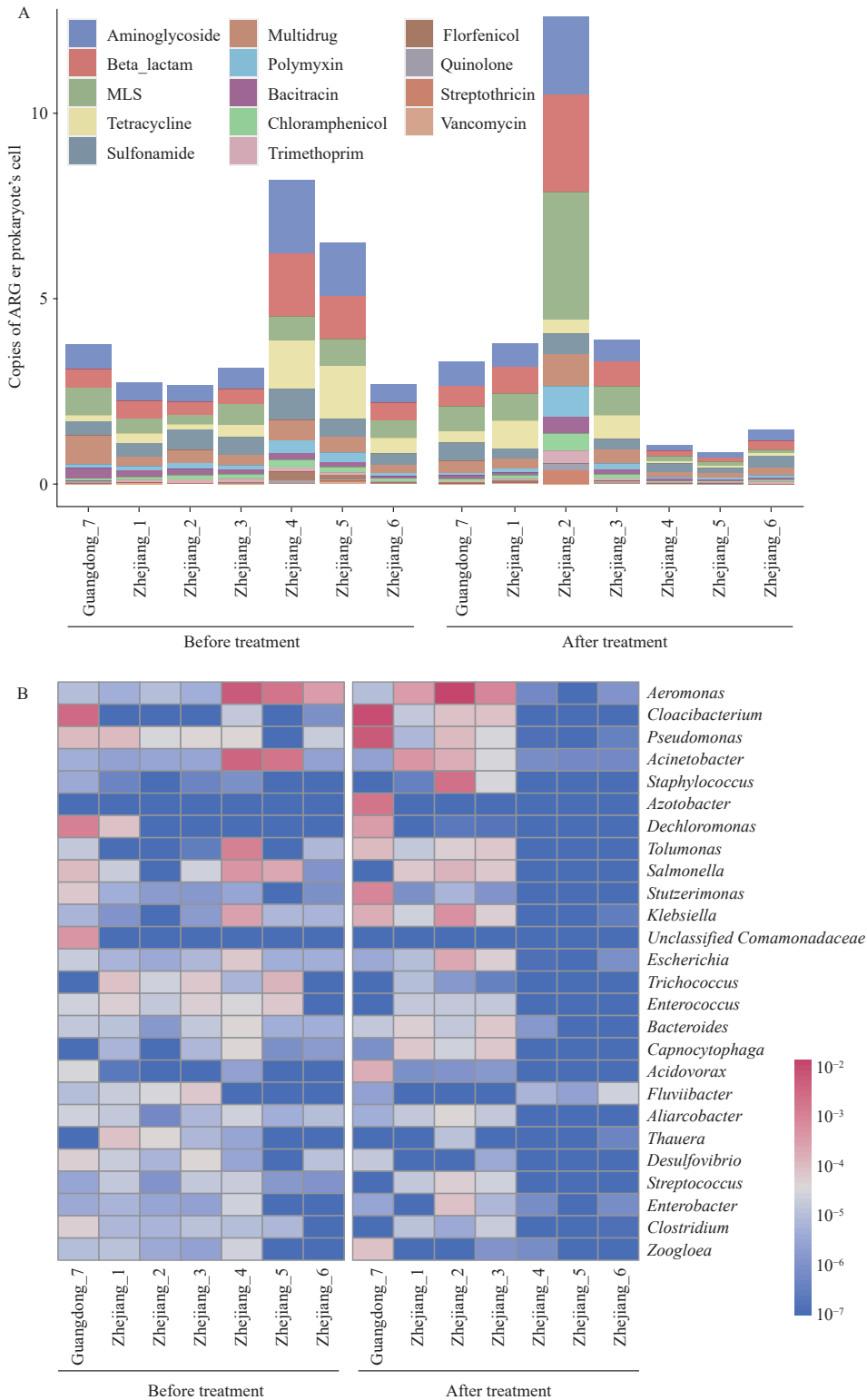
SUPPLEMENTARY FIGURE S2. The comparison of antibiotic resistance before and after treatment. (A) The abundance of ARGs before and after treatment. The ARG types whose average abundance ≥0.01 copies per cell were presented. (B) The relative abundance of ARG-carrying genera (average relative abundance ≥0.1%).
Note: The treatment for samples of Zhejiang_1, Zhejiang_2, and Zhejiang_3 was activated sludge process. The treatment for samples of Zhejiang_4, Zhejiang_5, and Zhejiang_6 was chlorination disinfection. The treatment for the sample of Guangdong_7 was UV disinfection.
Abbreviation: MLS=macrolide-lincosamide-streptogramin; ATGs=antibiotic resistance genes.

SUPPLEMENTARY TABLE S1. The detailed information of metagenomic datasets.

NCBI Project ID	Province	ID	Data size (Mb)	Note
PRJNA989503	Gansu	Gansu_1	3397.5	before treatment
		Gansu_2	3159.9	before treatment
		Gansu_3	3550.7	before treatment
		Gansu_4	3201.8	before treatment
		Gansu_5	3168.3	before treatment
		Gansu_6	3727.5	before treatment
		Gansu_7	3806.4	before treatment
		Gansu_8	3315.4	before treatment
		Gansu_9	3268.8	before treatment
		Gansu_10	3240.5	before treatment
		Gansu_11	3775.9	before treatment
		Gansu_12	3215.4	before treatment
		Gansu_13	3286.0	before treatment
		Gansu_14	3378.2	before treatment
		Gansu_15	3245.6	before treatment
		Gansu_16	3448.7	before treatment
		Gansu_17	3204.3	before treatment
		Gansu_18	3878.9	before treatment
		Gansu_19	3260.1	before treatment
		Gansu_20	3324.4	before treatment
		Gansu_21	3423.6	before treatment
		Gansu_22	3320.3	before treatment
		Gansu_23	3210.2	before treatment
		Gansu_24	3319.3	before treatment
		Gansu_25	3278.4	before treatment
		Gansu_26	3296.9	before treatment
		Gansu_27	3266.7	before treatment
		Gansu_28	3160.5	before treatment
		Gansu_29	3321.8	before treatment
		Gansu_30	4105.5	before treatment
PRJNA876047	Hebei	Hebei_1	2653.3	before treatment
		Hebei_2	2792.5	before treatment
		Hebei_3	2970.6	before treatment
		Hebei_4	2833.8	before treatment
		Hebei_5	2656.2	before treatment
		Hebei_6	2724.7	before treatment
		Hebei_7	2748.3	before treatment
		Hebei_8	2772.1	before treatment
		Hebei_9	2733.4	before treatment
		Hebei_10	2741.5	before treatment
		Hebei_11	2253.3	before treatment
		Hebei_12	2766.4	before treatment
		Hebei_13	2767.6	before treatment
		Hebei_14	3057.4	before treatment
		Hebei_15	2692.4	before treatment
		Hebei_16	2763.2	before treatment

TABLE S1. (Continued)

NCBI Project ID	Province	ID	Data size (Mb)	Note
PRJNA862690	Hunan	Hunan_1	2550.5	before treatment
		Hunan_2	2472.8	before treatment
		Hunan_3	2633.3	before treatment
PRJNA723368	Sichuan	Sichuan_1	4090.7	before treatment
		Sichuan_2	4154.7	before treatment
		Sichuan_3	4178.7	before treatment
		Sichuan_4	4086.4	before treatment
		Sichuan_5	4448.0	before treatment
		Sichuan_6	4203.4	before treatment
		Sichuan_7	4414.1	before treatment
		Sichuan_8	5001.2	before treatment
		Sichuan_9	4602.3	before treatment
PRJNA612238	Guangdong	Guangdong_1	2231.2	before treatment
		Guangdong_2	2361.1	before treatment
		Guangdong_3	2302.8	before treatment
		Guangdong_4	2645.6	before treatment
		Guangdong_5	2251.6	before treatment
		Guangdong_6	2759.7	before treatment
This study		Guangdong_7	9748.5	before treatment
		Guangdong_7	10086.4	after treatment
PRJNA770854	Zhejiang	Zhejiang_1	3051.5	before treatment
		Zhejiang_2	7300.7	before treatment
		Zhejiang_3	4646.1	before treatment
		Zhejiang_4	3712.8	before treatment
		Zhejiang_5	3571.9	before treatment
		Zhejiang_6	4548.1	before treatment
		Zhejiang_1	3910.9	after treatment
		Zhejiang_2	3711.8	after treatment
		Zhejiang_3	4037.6	after treatment
		Zhejiang_4	3537.1	after treatment
		Zhejiang_5	3245.9	after treatment
		Zhejiang_6	3293.7	after treatment