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Original Article

Monitoring and Homology Analysis of Extended-Spectrum Beta-Lactamases (ESBLS) Producing *Escherichia Coli* from the Hospital Sewage in Shaoxing, China

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Abstract:

Objective: To detect drug resistance and homology analysis of Escherichia coli isolated from hospital sewage, and to explore the sources of antibiotic-resistant microorganisms in the environment.

Methods: Thermotolerant *Escherichia coli* and other enteric pathogenic bacteria isolated from the sewage of three hospitals in Shaoxing area from January 2016 to December 2019 were included. The drug resistance of thermotolerant *Escherichia coli* was detected and compared with that of *Escherichia coli* isolated from clinical specimens in 2019. Meanwhile, the resistance phenotype and resistance genes of extended spectrum beta lactamases (ESBLs) of thermotolerant *Escherichia coli* in sewage were detected.

Results: A total of 65 strains of pathogenic bacteria were isolated from sewage, including 45 strains of *Escherichia coli*. *Escherichia coli* had a lower resistance rate to most antibiotics compared with *Escherichia coli* isolated from clinical specimens, and the resistance rates of *Ampicillin*, *Aztreonam*, *Amoxicillin*/*Clavulanic* acid and first to fourth generation *Cephalosporins* were statistically significant (P<0.05). A total of 20 ESBLs-positive strains were detected in sewage, including 10 strains carrying *CTX-M9* resistance gene, 8 strains carrying *CTX-M-1* resistance gene, 5 strains carrying *TEM* resistance gene. In addition, a pan-drug resistant *Klebsiella pneumoniae* was detected.

Conclusion: Intestinal pathogens carrying different drug resistance genes in hospital sewage. Hospitals should strengthen the quality of sewage treatment to prevent the spread of multi-drug resistant bacteria to the natural environment.

Keyword: Hospital sewage, Escherichia coli, Surveillance, Drug resistance, Homology

1. Introduction

Antibacterial drugs are widely used in medical and health care, agricultural breeding and other fields, and play an important role in the treatment of infectious diseases and ensuring public health security. However, due to the unreasonable use or even abuse of antibiotics, the resistance rate of pathogens to antibiotics has increased yearly[1]. On the other hand, most antibiotics cannot be completely metabolized in humans and animals and can be excreted in the form of active metabolites or even original drugs. These substances will eventually enter the water environment through a variety of ways such as hospital sewage, domestic sewage, livestock manure and pharmaceutical industrial wastewater[2, 3]. It has been found that drug-resistant bacteria exist in a variety of water bodies and have a tendency to increase the proportion of multi-drug resistance of microorganisms in the water environment[4, 5]. If unqualified hospital sewage is discharged into municipal sewage pipelines and natural water bodies, it will seriously pollute water sources, spread diseases and endanger people's health[6-8]. At present, there is no report on the distribution and drug resistance of pathogenic bacteria in the sewage of hospitals in this region. In order to understand the disinfection status of the sewage of medical units

in this region, this study carried out bacterial culture on the sewage submitted for inspection by three hospitals, and tested the drug sensitivity of the heat-resistant Escherichia coli isolated. At the same time, the drug resistance of Escherichia coli isolated in clinic was compared, so as to master the sewage disinfection status of hospitals in this area and provide reference for its management and facility construction.

Materials and Methods

Specimen collection

Sewage was collected from three hospitals in Shaoxing area from January 2016 to December 2019, including a tertiary hospital (A hospital), a secondary hospital (B hospital) and a community hospital (C hospital), once a month, for a total of 48 times. All hospitals adopted centralized secondary sewage treatment process, and the samples taken were all centralized treated sewage. Samples shall be taken according to the requirements of aseptic operation, and no less than 200 ml of samples shall be taken and placed in sterilized glass bottles, and delivered to the microbiology laboratory of our hospital before 12:00 am on the same day. *E. coli* was isolated from patients' blood, sputum, urine and drainage fluid in the three hospitals.

Isolation and culture of thermotolerant coliforms

Our test was slightly modified to detect the thermotolerant coliform bacteria in sewage according to the previously described methods[9]. First, the initial fermentation experiment was conducted. We selected three suitable sample homogenates of continuous dilution, including stock solution, 10-fold dilution and 100-fold dilution, and inoculated 1ml of each into lauryl sulfate tryptone (LST, Hangzhou Binhe Microbial Reagent Co., Ltd.) broth, and then cultured at about 44℃. We observed and recorded whether there are bubbles in the test tube or whether there are dense and continuous small bubbles escaping from the bottom of the tube when shaking the test tube at 24 h and 48 h.

Secondly, all gas producing or turbid tubes were tested for a re-fermentation experiment. 100 ul of the cultures were taken from all the LST broth tubes of 48 h fermentation gas production was transplanted into the broth tube of Brilliant Green Lactose Bile Broth (BGLB, Hangzhou Binhe Microbial Reagent Co., Ltd.), and incubated at 44℃ for 48 h. We identified gas producing or turbidity tubes as coliform positive tubes. According to the number of positive tubes of fecal coliform, the key table of the most probable number (MPN) of fecal coliform in sewage was checked, and the MPN value of fecal coliform in each liter of sample was converted and reported. Results > 500 MPN/L was unqualified.

Finally, the broth in the above turbidity tube was streaked into the Eosin methylene blue plate (Hangzhou Binhe Microbial Reagent Co., Ltd.) and incubated at 35℃. After 24 h, purple-black or lavender red colonies were selected for identification and drug sensitivity test by Vitek-2 bacterial identification instrument (Biomerieux, France), But drugs such as tigecycline, meropenem, cefuroxime and cefoperazone sulbactam were tested by disk diffusion. Meanwhile, the antibiotic sensitivity test and drug resistance gene detection were carried out for the dominant strains. Salmonella and Shigella diagnostic serum (Ningbo Tianrun Biopharmaceutical Co., Ltd) were used to identify Salmonella and Shigella, respectively.

Detection of drug resistance genes by Polymerase Chain Reaction (PCR)

Primers for drug-resistance genes were designed according to the literature, and the TEM, SHV, CTX and NDM genes of heat-resistant *E. coli* were detected[10, 11]. Electrophoresis at 110V for 20 min and the results were observed by gel imager. PCR products with a clear and single target band were sent to Shanghai Sangon Bioengineering Co., Ltd. for sequencing. The final sequencing results were compared on the http://blast.ncbi.nlm.nih.gov/Blast.cgi website and the corresponding enzyme types were determined. Whole genome sequencing was used to determine the resistance genes and virulence factors of a pan-drug-resistant *Klebsiella pneumoniae* strain.

Multi-locus sequence typing (MLST)

MLST typing of ESBLs positive strains was studied. Primer sequences of seven housekeeping genes used for MLST were downloaded from http://www.mlst.net/ website and synthesized by Shanghai Sangon Biotech Co., Ltd. The PCR products of the seven MLST-typed housekeeping genes tested were purified and sequenced. The numbers of alleles and sequence types were

assigned using MLST online database (https://cge.food.dtu.dk/services/MLST).

Results

Detection results of pathogenic bacteria

A total of 65 strains of pathogenic bacteria were detected from the sewage of three hospitals in Shaoxing area from January 2016 to December 2019. There were 45 strains of *Escherichia coli* (69.2%), mainly heat-resistant *Escherichia coli*, 2 strains of *Shigella sonnei* and 1 strain of *Salmonella arizona*. Among the unqualified tests of thermotolerant coliform bacteria in three hospitals, A hospital had one unqualified test (May 2019), B hospital had four unqualified tests (April, May, September 2016 and August 2017), and C hospital had nine unqualified tests (March, April, May, June, July, August, September 2016, November 2017 and June 2018, respectively). The pathogen species are shown in **Table 1**.

Table 1 Species of pathogenic bacteria in sewage

Organism	A Hospital	B Hospital	C Hospital	Total
Escherichia coli	3	12	30	45
Aeromonas hydrophila	1	1	3	5
Enterobacter cloacae	1	1	1	3
Middle Lüvol	0	1	2	3
Raoultella planticola	0	1	1	2
Enterobacter aerogenes	0	0	1	1
Enterobacter amnigenus	0	0	1	1
Citrobacter braakii	0	0	1	1
Klebsiella pneumoniae	0	1	0	1
Shigella sonnei	0	0	2	2
Salmonella arizonae	0	1	0	1
Total	5	18	42	65

Resistance of *Escherichia Coli*

The resistance rates of heat-resistant *Escherichia coli* in sewage to *tigecycline*, carbapenem antibiotics (*imipenem*, *meropenem*, *ertapenem*) and enzyme inhibitors (*amoxicillin* / *clavulanic acid*, *cefoperazone* / *sulbactam*, *piperacillin* / *tazobactam*) were 0, and the resistance rates to other antibiotics were less than 40%. The positive rate of ESBLs was 44.4% (20/45). Compared with the drug resistance rate of *Escherichia coli* detected in clinical specimens from three hospitals, the differences in the drug resistance rates of *aztreonam*, cephalosporins (*cefuroxime*, *ceftriaxone*, *ceftazidime*, *cefepime*), quinolones (*ciprofloxacin*, *levofloxacin*) and enzyme inhibitors (*amoxicillin* / *clavulanic acid*, *cefoperazone*/*sulbactam*) were statistically significant ($P < 0.05$). These results were shown in **Table 2**.

Table 2 Comparison of drug resistance rate of *E. coli* in hospital sewage and clinical specimens

Antibiotics	Hospital sewage(n=45)		Clinical specimens(n=1114)		χ^2	P
	Case	Drug resistance rate	Case	Drug resistance rate		
Tigecycline	0	0	2	0.2	0.081	0.776
Amikacin	0	0	24	2.2	0.99	0.320
Imipenem	0	0	33	3.0	1.372	0.241
meropenem	0	0	39	3.5	1.63	0.202
Ertapenem	0	0	39	3.5	1.63	0.202
Macrodantin	0	0	54	4.8	2.288	0.130
Amoxicillin /	0	0	165	14.8	7.772	0.005

clavulanic acid						
Cefoperazone / sulbactam	0	0	105	9.4	4.664	0.031
Cefoperazone / sulbactam	0	0	53	4.7	2.244	0.134
ceftazidime	0	0	270	24.2	14.219	0.000
cefepime	0	0	132	11.8	6.017	0.014
tobramycin	4	8.9	123	11.0	0.205	0.650
cefoxitin	5	11.1	177	15.9	0.746	0.388
aztreonam	6	13.3	381	34.2	8.468	0.004
levofloxacin	8	17.8	530	47.6	15.442	0.000
ciprofloxacin	9	20.0	546	49.0	14.589	0.000
cefuroxime	9	20.0	633	56.8	23.734	0.000
ceftriaxone	15	33.3	605	54.3	7.649	0.006
selectrin	18	40.0	531	47.7	1.02	0.313
cefazolin	20	44.4	649	58.3	3.382	0.066
ampicillin	28	62.2	896	80.4	8.872	0.003

Drug resistance Gene Detection

Among the 20 ESBLs positive heat-resistant *Escherichia coli*, 9 strains carried *CTX-M9* resistance gene, 8 strains carried *CTX-M-1* resistance gene, 5 strains carried *TEM* resistance gene, 2 strains carried both *CTX-M9* and *TEM* resistance genes, 1 strain carried both *CTX-M9* and *SHV* resistance genes, and 1 strain carried both *CTX-M9* and *DHA* resistance genes. MLST was divided into 12 types, and the dominant type was ST167 (4 strains), which was distributed in hospital A and B, as shown in **Table 3**.

Table 3 Genotypes, MLST typing and hospital distribution of 20 ESBLs positive strains

No	Genotype	MLST	Hospital
1	SHV、***CTX-M-9***	10	B
2	CTX-M-1	10	B
3	CTX-M-9	48	B
4	CTX-M-1	93	B
5	CTX-M-9	101	C
6	TEM, ***CTX-M-9***	101	C
7	TEM, ***CTX-M-9***	155	C
8	TEM	167	A
9	CTX-M-9	167	A
10	TEM	167	B
11	CTX-M-1	167	B
12	CTX-M-1	215	B
13	CTX-M-2	224	C
14	CTX-M-1	297	C
15	CTX-M-9	354	C
16	TEM, ***CTX-M-1***	354	C
17	CTX-M-1	405	B
18	CTX-M-9	410	C
19	CTX-M-1	410	C
20	CTX-M-9	410	C

In addition, a pan-resistant *Klebsiella pneumoniae* strain was discovered. After sequencing, it was

found that it carried β-lactam resistance genes such as *SHV-28*, *DHA-18*, *NMC*, *SPM*, *AIM*, and lacked efflux systems such as AcrA-AcrB-TolC and OmpK35 and OmpK36 outer membrane proteins. In addition, it contains virulence factors including iron uptake system, fimbriae, capsule, endotoxin, binary regulation system and so on. The MLST typing of *klebsiella pneumonia* was ST15.

Discussion

Thermotolerant coliform bacteria is one of the important indexes to evaluate the quality of hospital sewage, which is of great significance in hygiene. It belongs to a subspecies of total coliform bacteria and comes from feces. In addition to heat resistance, thermotolerant coliforms can still grow and reproduce under high temperature conditions of 44 - 44.5℃ and metabolize tryptophan into indole, and other characteristics are the same as total coliforms. The sanitary quality of hospital sewage was evaluated by monitoring the thermotolerant coliform bacteria in sewage. Water environment is one of the important convergence carriers of various pollutants, and urbanization of water areas has enhanced the aggregation of pathogenic bacteria and antibiotic resistance genes in the water environment[12]. The remaining antibacterial drugs are poured into the sewer, which leads to the direct exposure of antibacterial drugs to the natural environment and sewage. The sewage is full of intestinal bacterial pathogens, which are exposed to antibiotics and other cell bodies containing antibiotic resistance genes, and these genes can spread horizontally, resulting in the spread of drug-resistant bacteria[13]. Therefore, if the hospital sewage is not strictly disinfected and purified, it will seriously pollute the living environment and become a new way for the spread of drug-resistant bacteria infection[14].

In this study, the detection and drug resistance of heat-resistant coliforms in sewage from three hospitals in Shaoxing area were analyzed. The results showed that more than ten kinds of pathogenic bacteria were detected in the three hospitals. Among them, the detection rate of *Escherichia coli* was the highest, accounting for 69.2%, and its detection rate ranking was consistent with that of clinical specimens[1]. The second is *Aeromonas hydrophila*, which is widely distributed in various water bodies in nature and is the primary pathogen of a variety of aquatic animals. It can produce highly toxic exotoxins, which can lead to endogenous blood, abdominal cavity, biliary tract, wound or urinary tract infection[15]. It is worth noting that two strains of *Shigella sonnei* and one strain of *Salmonella arizona* were also detected in the sewage. *Shigella* can cause bacillary dysentery, and in severe cases can lead to inflammation, necrosis and ulceration of the intestinal mucosa[16]. *Salmonella* can cause typhoid fever, paratyphoid fever and acute gastroenteritis, and food poisoning can be caused by fecal contamination of food from people or carriers infected with *Salmonella*[17]. These pathogens can pollute plants and food through water environment, which will bring great threat to human health.

Compared with the clinically detected pathogens, the thermotolerant *E. coli* detected from sewage had a lower resistance rate to most antibiotics, and no carbapenem-resistant *E. coli* was detected. The resistance rate of some antibiotics was significantly lower than that of *E. coli* detected in clinical specimens ($P < 0.05$), it is possible that the resistance of bacteria to antibiotics has been changed after the disinfection. The molecular biology analysis of 20 ESBLS-positive *E. coli* strains showed that multiple drug resistance genes were detected in all ESBLS-positive strains, and 4 strains had two drug resistance genes simultaneously. There were 12 ST types of ESBLs-positive *E. coli*, and the dominant type was ST167 (4 strains), which was distributed in A and B hospitals. This result was significantly different from the ST typing of ESBLs-positive *E. coli* detected in clinical specimens in the province. ST131 was the main type of clinical samples, while ST167 belonged to a few types, indicating that *E. coli* isolated from sewage and clinical specimens may be derived from different clones[18]. In addition, the two strains of *E. coli* from A hospital belonged to ST167 type, but carried different drug resistance genes. The eight strains of *E. coli* from hospital B and the 10 strains of *E. coli* from C hospital belonged to six different ST types respectively, and their drug resistance genes are also different. It was indicated that the molecular epidemiological characteristics of thermotolerant *E. coli* detected in sewage in this area are diverse.

Moreover, a pan-resistant Klebsiella pneumoniae

strain was detected in sewage. After sequencing, it was found to carry multiple β-lactam resistance genes such as *SHV-28* and *DHA-18*, as well as OmpK35 / OmpK36 outer membrane protein deletion, and a variety of virulence factors were also detected. After discharge, these bacteria containing antibiotic resistance genes will spread to the natural environment through water flow, which will lead to the spread of drug-resistant bacteria[13]. It has been reported that multi-drug-resistant *E. coli* has been detected in the sewage of a hospital in India and can be horizontally transferred through plasmids[19].

According to the relevant literature, the phenomenon of substandard sewage discharge is still common in domestic hospitals[20-22]. The reasons include the lack of effective management of sewage treatment facilities, the non-standard operation of sewage treatment personnel, the untimely maintenance of sewage treatment equipment, the backward detection technology and the lack of advanced disinfection treatment methods. In recent years, there have been many studies on the detection of antibiotics or drug resistance genes in sewage, including the detection of antibiotic resistance genes in water environment by electrochemical sensors based on glassy carbon electrodes, the detection of quinolone antibiotic residues in water environment based on optical fiber integrated silver nanoparticle technology and the determination of antibiotic concentration in hospital sewage by solid phase extraction, liquid chromatography / mass spectrometry and analogue internal standard method[19, 23, 24]. Due to the long-term discharge of antibiotics in the aquatic environment, antibiotics can be detected in almost every environmental matrix. Therefore, the removal of antibiotics from polluted aquatic environment has aroused great interest of researchers. At present, several techniques have been used to remove the pollution of antibiotics in water, including the use of cationic surfactant functionalized nano silica rice husk, a variety of technology has become the current research focus[25-27].

It is worth mentioning that since 2017, Shaoxing Center for Disease Control and Prevention has strengthened the sampling survey of sewage discharge quality of medical units in the region, and notified and punished units that did not meet the standards. Therefore, since 2017, various medical units in Shaoxing have actively introduced new technologies, strengthened the requirements for sewage discharge detection, and the phenomenon of non-compliance has been greatly reduced.

Conclusion

To sum up, there are different kinds of intestinal pathogenic bacteria in hospital sewage in this area, which may lead to the pollution of drug-resistant microorganisms in the environment. Hospitals should strengthen the supervision of sewage treatment, improve the sewage treatment process, learn from new technologies and methods in other regions or hospitals, and establish normalized sewage hygiene monitoring norms, so as to prevent the spread of multi-drug-resistant bacteria to the natural environment.

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Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by LR, ML and QH. the first draft of the manuscript was written by LR. Data Curation and Validation and were performed by XZ and YW. Project administration and Validation were performed by MH. Supervision, Reviewing and Editing were performed by GM. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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