

Original Article

Antimicrobial resistance and integrons of ESBL-producing thermotolerant coliforms from a water reservoir in Tai'an, China

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Abstract

Introduction: The contamination of water environments by extended-spectrum β -lactamases-producing thermotolerant coliforms (ESBL-TC) has aroused public concern. However, little epidemiological data on ESBL-TC isolates from water reservoirs is available in China.

Methodology: This study was designed to investigate antibiotic resistance, *bla* gene types, and the presence of integrons (class 1, 2, and 3) and gene cassettes in ESBL-TC isolated from the Huangqian Reservoir of Tai'an, China.

Results: A total of 96 non-duplicate ESBL-TC were obtained in this study and the ESBL genes included *bla*_{CTX-M-14} (n = 47), *bla*_{CTX-M-15} (n = 27), *bla*_{CTX-M-55} (n = 18), *bla*_{SHV-12} (n = 4), *bla*_{CTX-M-3} (n = 3), and *bla*_{CTX-M-123} (n = 1). Eighty-three of the ninety-six ESBL-TC contained class 1 integrons (86.5%), and 2 isolates harbored class 2 integrons. The sizes of gene cassette regions within integrons were ranged from 0.2 kb to 3.2 kb.

Conclusions: The findings of this study indicated the widespread presence of ESBL-TC strains in the Huangqian Reservoir and spotlighted the potential role of water bodies as reservoirs for antibiotic resistant genes.

Key words: Thermotolerant coliform; integron; water reservoir; ESBL; antibiotic resistance genes.

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Introduction

The long-term use of antibiotics in clinical practices has led to the occurrence of antimicrobial resistant bacteria in various environments [1,2]. It is believed that drug resistant bacteria from humans and animals, discharged into water bodies, could result in the spread of resistance genes within the bacterial community [3-7]. Therefore, the persistence of antibiotic-resistant intestinal bacteria in water environments, particularly extended-spectrum β -lactamase (ESBL)-producing bacteria, could elicit an important public health risk [8,9].

ESBL-producing thermotolerant coliforms (ESBL-TC) belong to *Enterobacteriaceae* family and come chiefly from fecal flora of human beings and other warm-blooded animals [10]. Once entering into water bodies, ESBL-TC strains could contribute to the dissemination of antibiotic resistance genes within bacterial community via mobile genetic elements (MGE) [11]. The integron is an important MGE member that can mediate the capture and integration of gene cassettes mainly encoding antibiotic resistance

[12]. Importantly, the integron system can import different combinations of antibiotic resistance genes, leading to the appearance of multi-drug resistant (MDR) bacteria [13,14].

Numerous studies have investigated ESBL-producing bacteria from various water environments such as: rivers, lakes, springs, wells, and sewage treatment plants [15-18]. The Huangqian Reservoir is the main drinking and irrigation water source for the city of Tai'an, China; the microbial quality of its water is associated with the health of 1.7 million inhabitants. However, the background information of ESBL-producing bacteria in the Huangqian Reservoir remains poorly understood to date. The aim of this study was to investigate the occurrence and characterization of ESBL-TC in the Huangqian Reservoir.

Methodology

Sampling and processing of water samples

Between October and December 2014, water samples were collected from the Huangqian Reservoir of Tai'an of China (36.32N, 117.25E). A total of 100

water samples (100 mL water per sample) were collected from within 10 m of 5 different floodgates (20×5) using sterile containers. After collection, water samples were transported in a cooler to our microbiological lab and processed within 4-6 hours.

Isolation and identification of ESBL-TC

Each water sample was filtered through membrane filters with a pore size of 0.45 µm (Millipore, Billerica, USA). The filters were incubated for 24 hours at 44.5 °C in 10 mL of Enterobacteria Enrichment Broth (Haibo, Qingdao, China). One loopful of each of the enrichment cultures was inoculated onto chromogenic Brilliance ESBL agar (Oxoid, Hampshire, UK) to select for ESBL-producers [5,19,20]. All suspected ESBL-producing bacteria were re-selected by MacConkey agar containing cefotaxime 64 mg/mL (Haibo, Qingdao, China) [11]. All cefotaxime resistant bacteria were identified by the automated microbiology system Vitek 2 Compact (bio-Mérieux, Marcy l'Etoile, France). These isolates were further tested for ESBL production by double disk diffusion methods using cefotaxime (30 mg), ceftazidime (30 mg) and clavulanic acid (10 mg) according to the Clinical and Laboratory standards institute guidelines [21].

Clonal analysis by ERIC-PCR

As described previously, enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR) was used to determine clonal relatedness of ESBL-TC [22]. Briefly, amplification was conducted as follows: an initial denaturation (94°C, 5 minutes), followed by 35 cycles of denaturation (94°C, 1 minute), annealing (42°C, 1 minute), and extension (65°C, 8 minutes) with a single final extension (65°C, 16 minutes). The reaction products were electrophoresed on a 1.5% agarose gel. NTSYS-pc (version 2.02 K, Applied Biostatistics, Inc., NY, USA) was used to construct a dendrogram based on the average similarity of the matrix [23]. Clonal relatedness was assigned based on > 80% similarity [24].

Antimicrobial susceptibility testing

The susceptibility of ESBL-TC to 17 antimicrobials was performed by disc diffusion method [21]. The antimicrobials used in this study were cefazolin, cefaclor, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, cefepime, aztreonam, ampicillin, piperacillin, ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole, gentamicin, amikacin, imipenem and meropenem (Becton Dickinson, Sparks, USA). An isolate was considered multidrug-resistant (MDR)

when exhibiting resistance to antibiotics of at least three different classes [25]. *Escherichia coli* ATCC 25922 was used as the control strain in this study.

PCR to detect bla genes

According to previously published references, PCR was performed to detect *bla*_{TEM} [26], *bla*_{SHV} [27], and *bla*_{CTX-M}. *bla*_{CTX-M} group-specific primers for CTX-M-1 [28], CTX-M-2 [28], CTX-M-8 [26] and CTX-M-9 [29,30] groups were used for the detection of *bla*_{CTX-M} genes. PCR products were separated by electrophoresis using a 1% agarose gel to identify the amplified DNA fragments. Amplicons were sequenced and searched against Genbank using the BLAST alignment tool (<http://www.ncbi.nlm.nih.gov/blast/>) to identify the resistance gene allele.

Detection of integrase genes and characterization of gene cassettes

Class 1, 2 and 3 integrase genes were amplified by multiplex PCR as described previously [11,31]. Briefly, Conditions for amplification were as follows: 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds; 54°C for 30 seconds; and 72°C for 120 seconds, with a final extension step at 72°C for 10 minutes. *Vibrio cholerae* O₁ strain SK10, *E. coli* DH5a with pR483:Tn7, *E. coli* DH5a with pSMB731 were used as positive controls for class 1, 2 and 3 integrons, respectively. *E. coli* C600 was used as negative control.

The presence of integrons was assessed by PCR using specific primers hep58 and hep59 for the gene cassette regions of class 1 and primers shep51 and hep74 for class 2 integrons [32,33]. ESBL-TC isolates with identical PCR-restriction fragment length polymorphism (PCR-RFLP) patterns were considered as the same gene cassette array [11]. The PCR products of different band sizes were sequenced and analyzed using the BLAST and ORF finder programs provided on the NCBI website.

Results

Isolation and identification of ESBL-TC

A total of 981 TC were isolated from the Huangqian Reservoir, and 126 (126/981, 12.8%) ESBL-TC isolates were confirmed by the double disk diffusion method. After clonal analysis by ERIC-PCR, 96 non-duplicate ESBL-TC were obtained in this study.

Of the 96 non-duplicate ESBL-TC isolates, *E. coli* constituted the largest portion (52/96, 54.2%), followed by *Klebsiella pneumoniae* (26/96, 27.1%), *Enterobacter cloacae* (8/96, 8.3%), *Pantoea* spp (4/96,

4.2%), *Citrobacter freundii* (3/96, 3.1%), *K. oxytoca* (2/96, 2.1%), and *Serratia odorifera* (1/96, 1.0%).

Antimicrobial susceptibility

All 96 non-duplicate ESBL-TC strains were resistant to the first- and second-generation cephalosporin antibiotics, such as cefazolin, cefaclor and cefuroxime. The resistance rates to third-generation cephalosporins were as follows: cefotaxime (92/96, 95.8%) ceftriaxone (92/96, 95.8%) and ceftazidime (32/96, 33.3%). Resistance rate to cefepime (the fourth-generation cephalosporin) was 12.5% (12/96). Resistance rate to aztreonam (monobactams antibiotic) was 26.0% (25/96). High resistance rates to non-cephalosporin were observed in this study, such as ampicillin (93/96, 96.9%), piperacillin (90/96, 93.8%), ciprofloxacin (82/96, 85.4%), tetracycline (80/96, 83.3%), trimethoprim-sulfamethoxazole (72/96, 75.0%) and gentamicin (70/96, 72.9%). Of note, few isolates were resistant to amikacin (7.3%), and all isolates were susceptible to imipenem and meropenem (Figure 1).

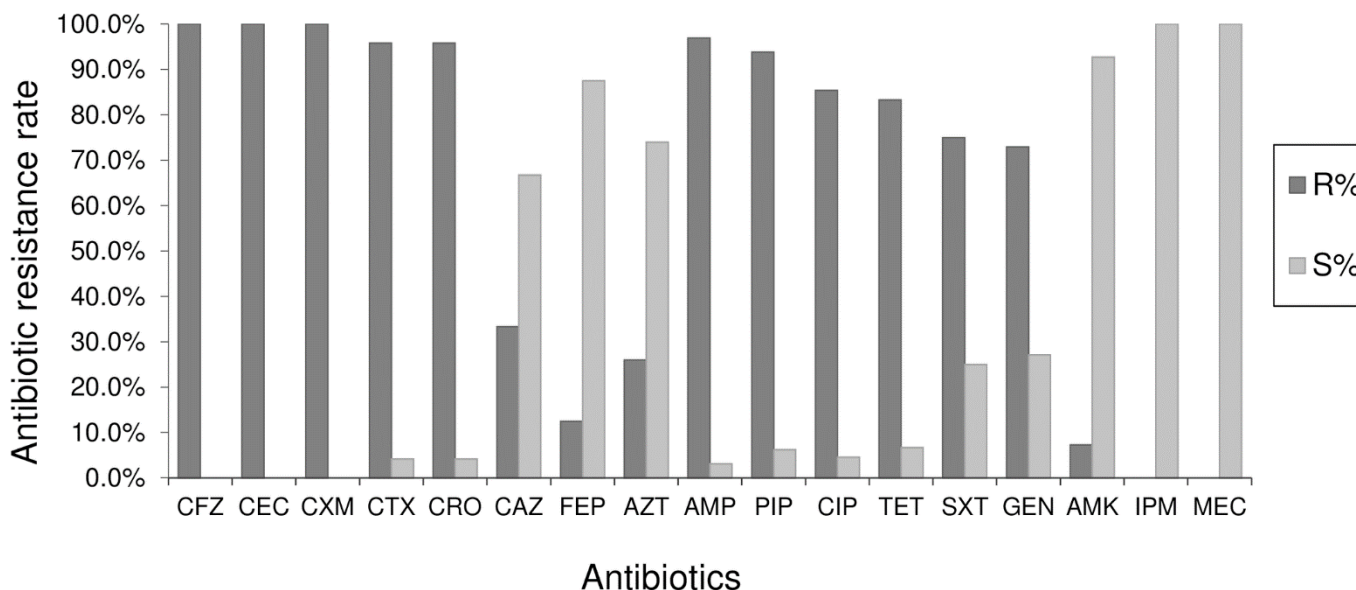
β-lactamase genes

All 96 non-duplicate ESBL-TC strains contained ESBL genes. Eighty-four of 96 ESBL-TC isolates carried more than one β-lactamase gene, including the combination of *bla*_{TEM-1+CTX-M} genes constituting the largest portion of combined *bla* gene types, and the

Table 1 β-lactamase distribution in 96 ESBL-TC isolates.

β-lactamase type	Strains carrying the gene (s)	No. of isolates
CTX-M-14	<i>E. coli</i>	6
	<i>K. pneumoniae</i>	3
	Total	9
CTX-M-15	<i>E. coli</i>	1
	<i>K. pneumoniae</i>	2
	Total	3
CTX-M-14 +TEM-1	<i>E. coli</i>	13
	<i>Pantoea spp</i>	4
	<i>K. pneumoniae</i>	21
	Total	38
CTX-M-15 +TEM-1+SHV-12	<i>E. coli</i>	4
	Total	4
CTX-M-15 +TEM-1	<i>E. coli</i>	12
	<i>Enterobacter cloacae</i>	8
	Total	20
CTX-M-55 +TEM-1	<i>E. coli</i>	16
	<i>K. oxytoca</i>	2
	Total	18
CTX-M-3 +TEM-1	<i>Citrobacter freundii</i>	3
CTX-M-123 +TEM-1	<i>Serratia odorifera</i>	1

Figure 1. Antibiotic resistance characteristics of ESBL-TC isolates in this study.



R%, resistance rate; S, susceptible rate; CFZ, cefazolin; CEC, cefaclor; CXM, cefuroxime; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; AZT, aztreonam; AMP, ampicillin; PIP, piperacillin; CIP, ciprofloxacin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; GEN, gentamicin; AMK, amikacin; IPM, imipenem; MEC, meropenem

combination of *bla*_{TEM-1+CTX-M-14+SHV-12} genes (4). Twelve isolates contained a single *bla* gene, including *bla*_{CTX-M-14} (9), and *bla*_{CTX-M-15} (3) (Table 1).

Detection of integrase genes and characterization of gene cassettes

Of 96 non-duplicate ESBL-TC, 83 contained class 1 integrons (83/96, 86.5%); Class 2 integrons were only observed in two ESBL-producing *E. coli*. No class 3 integron was detected in this study. Among the 83 class 1 integron-positive isolates, 72 (72/83, 86.7%) carried gene cassettes, and 11 had empty integron (180 bp variable region) [20]. The sizes of gene cassette regions in class 1 integrons were between 0.2 kb to 3.2 kb. A 1.8 kb amplicon was the most frequently observed gene cassette region within class 1 integrons (33/72, 45.8%). There were 6 strains carrying more than one gene cassette region: 0.2 kb + 2.2 kb in three isolates and 1.8 kb + 2.4 kb in three isolates. Additionally, no gene cassette was detected in a 0.2 kb amplicon. Two class 2 integron-positive isolates harbored a 1.8 kb gene cassette region and a 2.4kb, respectively (Table 2).

Discussion

Water environments are the final storage container for wastewater of various kinds, such as hospitals, sewage treatment plants, and animal farms [34]. Therefore, it is important to investigate the prevalence and characteristics of ESBL-TC in natural water bodies; especially the city-supporting water sources. In the present study, a total of 126 (126/981, 12.8%) ESBL-TC were obtained from the the Huangqian Reservoir, with the dominant species being *E. coli* (52/96, 54.2%) and *K. pneumoniae* (26/96, 27.1%).

All 96 non-duplicate ESBL-TC strains were resistant to the first- and second-generation cephalosporins, cefazolin, cefaclor and cefuroxime, and were more resistant to the third-generation cephalosporins, cefotaxime (95.8%) and ceftriaxone (95.8%), than ceftazidime (33.3%). In addition, the resistance rate to non-cephalosporins was greater than 70%. These results showed an increasing trend in antibiotic resistance of water-borne ESBL-TC, compared to a previous investigation conducted in China [11]. The trend may mainly be due to the

Table 2 Distribution of gene cassette arrays found in class 1 and 2 integrons in ESBL-TC isolates.

Length of amplicon (kb)	<i>intI</i> gene	Gene cassette array	Strains carrying the gene cassette (s)	No. of isolates
0.2	1	nd ^a	<i>E. coli</i>	8
			<i>K. pneumoniae</i>	1
			Total	9
1.0	1	<i>aadA2</i>	<i>E. coli</i>	2
			<i>K. pneumoniae</i>	1
			Total	3
1.8	1	<i>dfrA17-aadA5</i>	<i>E. coli</i>	23
			<i>K. pneumoniae</i>	9
			<i>Citrobacter freundii</i>	1
Total	33			
1.8	2	<i>dfrA17-aadA5</i>	<i>E. coli</i>	1
2.2	1	<i>aac(6')-Ib-aar3-dfr27</i>	<i>E. coli</i>	7
			<i>K. pneumoniae</i>	3
			Total	10
2.4	1	<i>aacA4-cmlA1</i>	<i>E. coli</i>	5
			<i>K. pneumoniae</i>	4
			Total	9
2.4	2	<i>dfrA1-sat1-aadA1</i>	<i>E. coli</i>	1
3.2	1	<i>aadB-aadA1-cmlA6</i>	<i>E. coli</i>	2
0.2+2.2	1	<i>aac(6')-Ib-aar3-dfr27^b</i>	<i>E. coli</i>	3
1.8+2.4	1	<i>dfrA17-aadA5</i> and <i>aacA4-cmlA1</i>	<i>E. coli</i>	2
			<i>K. pneumoniae</i>	1
			Total	3

^aNot detected; ^b0.2 kb amplicon not included.

extensive use of third-generation cephalosporins in the clinical practices of China [30]. Of note, only the 7.3% of water-borne ESBL-TC were resistant to amikacin, and no isolate was resistant to imipenem and meropenem, which may be associated with the relatively low use in the local clinical practices.

CTX-M-14 (CTX-M-9 group) was the most common ESBL genotype detected in this study, followed by CTX-M-15 and CTX-M-55, which both belong to the CTX-M-1 group. CTX-M-15 differs from CTX-M-55 by a single amino acid substitution of Ala-77-Val. CTX-M-55 was first detected in clinical isolates of *E. coli* and *K. pneumoniae* from Thailand in 2005, and was subsequently found in *E. coli* and *Salmonella* spp. in China [30]. The *bla*_{CTX-M-3} gene was frequently detected in Chinese hospitals [30,35]. The *bla*_{CTX-M-123} gene, a hybrid of *bla*_{CTX-M-1} and *bla*_{CTX-M-9} group genes, was first isolated from diseased animals in Guangdong province of China in 2010 [36]. SHV-12 was first reported in China, while a subsequent large-scale study of isolates from six provinces, collected between 1998 and 2002, reported SHV-12 as the most common SHV type (ten isolates), with much smaller number of SHV-5, -2 and -9 [28]. The *bla*_{CTX-M} gene was the predominant ESBL-encoding gene in this study, which is in agreement with previous study performed in Chongqing city in China [11]. However, 84 out of 96 non-duplicate ESBL-TC contained *bla*_{TEM-1+CTX-M} genes (87.5%), which was higher than prior studies about ESBL-producing isolates from patients and animals in China [30,37]. The results indicated that *bla* genes from different sources may have exchanged and recombined in water bodies, leading to the predominance of *bla*_{CTX-M+TEM-1} phenotype of ESBL. In addition, the existence of *bla* genes in waters would become a public risk due to the fact that these genes can be spread to other water-borne microorganisms or even pathogens via MGEs and that ESBL-producers in human-associated water environments may pose serious health risks via drinking or the food chain [38,39].

Of 96 non-duplicate ESBL-TC; 83 (83/96, 86.5%) contained class 1 integrons and class 2 integrons were only observed in two ESBL-producing *E. coli*. For the remainder, we did not obtain PCR products, indicating altered sequences or the lack of *sulI* gene in the 3' conserved region of class 1 integrons [40]. The detection frequency of class 1 is much higher compared with numerous previous studies concerning ESBL-producing bacteria isolated from water environments [11,41]. By contrast, no 3 integrons were observed in the current study. These results further suggested that

class 1 integrons could easily transfer among ESBL-producing bacteria [42,43].

Conclusions

Collectively, the results of this study showed the Huangqian Reservoir is heavily contaminated by ESBL-TC isolates and suggest a potential role of water body as a reservoir for antibiotic resistant genes and the need of a long-term monitoring of antibiotic resistant bacteria in water environments.

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