Association of antibiotic resistance with SHV-12 extended-spectrum β-lactamase in *Enterobacter cloacae*

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Abstract. The association between antibiotic resistance and SHV-12 extended-spectrum β-lactamase (ESBL) in *Enterobacter cloacae* remains unknown. The aim of the present study was to investigate the prevalence of both chromosome- and plasmid-borne SHV-12 ESBL genes in *Enterobacter cloacae*. Transmission of the SHV-12 ESBL gene was explored, and the risk factors for antibiotic resistance in *E. cloacae* were analyzed. Polymerase chain reaction (PCR) results showed that 58 out of the 100 isolates carried the SHV-12 ESBL gene: 34.48% of them occurred in the chromosome, 48.28% were plasmid-borne and 17.24% appeared in both. Enterobacterial repetitive intergenic consensus-PCR tests detected 82 chromosomal genotypes. Conjugation assays showed that 70.00% of plasmid-borne SHV-12 ESBL genes were successfully transconjugated into *E. coli* C600 and that the antibiotic resistance phenotype of *E. cloacae* was partially (84%) or completely (10%) transferred. A significantly higher SHV-12 ESBL detection rate was found in patients with underlying conditions and/or complications compared with those without ($P<0.05$). The detection of SHV-12 ESBL-producing *E. cloacae* from vertical transmission varied significantly across clinical departments and age groups ($P<0.05$), with the highest rates in the intensive care unit and the group of patients aged ≥60 years. The present results indicate that the location and transmission efficiency of SHV-12 ESBL are closely correlated with the antibiotic resistance of *E. cloacae*.

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Introduction

Over recent decades, the *Enterobacter cloacae* complex (consisting of *Enterobacter cloacae, Enterobacter asburiae, Enterobacter hormaechei, Enterobacter kobei, Enterobacter ludwigii and Enterobacter nimipressuralis*) has taken on clinical significance, with its species emerging as nosocomial pathogens, particularly in intensive care units (ICUs). *E. cloacae* is the most representative species of the *E. cloacae* complex (1). This species can cause severe opportunistic infections in hospital patients, including lower respiratory, urinary tract and wound infections, as well as hospital-acquired sepsis (2). The increasing prevalence of *E. cloacae* is due predominantly to a high level of resistance to antimicrobial agents. SHV-type extended-spectrum β-lactamases (ESBLs), encoded by the bla_{SHV} gene confer significant resistance to β-lactam antibiotics (3). SHV-type ESBLs have >140 variants, and the SHV-12-type ESBL is one of the most prevalent types in Asia (4,5).

SHV-12 ESBL can be located on the chromosome, a plasmid or both (6) and can be transmitted horizontally and/or vertically. The location of the SHV-12 ESBL gene and the pathway of transmission vary by region, hospital and time span (7,8).

Bacteria continuously adjust gene expression in response to environmental changes. Currently, central venous catheters, malignant tumors, history of antibiotic usage and Pitt bacteremia score are regarded as the risk factors for bacterial infection (9); however, associated risk factors are distinct based on differences in hospital settings, practitioners, usage of medications and the condition of the patient, suggesting that additional external factors may be at play in the evolution of antibiotic resistance for *E. cloacae* (10-12).

Currently, the association between SHV-12 ESBL and the antibiotic resistance of *E. cloacae* has not been reported in the Guangdong region of China. In this study, therefore, the prevalence of *E. cloacae* and the correlation between the antibiotic resistance of *E. cloacae* and the prevalence status of SHV-12 ESBL were analyzed, a transmission model was delineated, and external factors that affect the location and transmission of SHV-12 ESBL were investigated. By

analyzing the association between the SHV-12 ESBL gene and the antibiotic resistance of *E. cloacae* with the combined effect of both internal and external factors, the aim was to provide evidence for the proper usage of antibiotics to help control the spread of drug-resistant genes.

Materials and methods

Bacterial isolates. Unique isolates of *E. cloacae* (n=100) were collected from randomly selected inpatients (62 men and 38 women) from the Affiliated Hospital of Guangdong Medical College (Zhanjiang, China) and the Taiping People's Hospital of Dongguan (Dongguan, China) between June 2012 and July 2013. All patients provided informed consent. Bacteria were identified using the BD Phoenix Automated Microbiology System (BD Pheonix™ 100; BD Biosciences, Franklin Lakes, NJ, USA) and the corresponding identification card. *Escherichia coli* ATCC 25922, *E. coli* C600 and SHV-12 ESBL-negative and -positive strains were from the Laboratory of Pathogenic Biology of Guangdong Medical College.

Antimicrobial susceptibility. Antimicrobial susceptibility testing was performed on 100 isolates by agar dilution, as recommended by the Clinical and Laboratory Standards Institute (13). The antibiotics were β-lactams [ampicillin (AM), cefazolin, cefuroxime (CXM), ceftazidime (CAZ), cefepime, cefoxitin (FOX), aztreonam (AZT), sulbactam + cefoperazone (SCF), tazobactam + piperacillin (TZP), imipenem (IMP), fluoroquinolone [ciprofloxacin (CIP) and levofloxacin (OFL)], aminoglycosides [amikacin (AMK) and gentamycin (GEN)] and a sulfonamide [sulfamethoxazole (SXT)]. All antibiotics were purchased from the National Institute for Food and Drug Control (Beijing, China)

Sequencing of SHV-12 ESBLs. The plasmid was eliminated using the variable-temperature-sodium dodecyl sulfate plasmid elimination method as described previously (14). Plasmid removal was verified using agarose gel electrophoresis. Chromosomal DNA was prepared using the FastPure™ DNA kit [Takara Biotechnology (Dalian) Co., Ltd., Dalian, China] and plasmid DNA was prepared using the Plasmid Mini kit (Qiagen, Hilden, Germany) following the manufacturers' instructions. The purified DNA was used as a template for PCR using an upstream primer pair, SHV-12-U (5'-GGTTATGCGTTATATTCGCC-3'), and a downstream primer pair, SHV-12-D (5'-TTAGCGTTGCCAGTGCTC-3'), which were synthesized in Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The desired polymerase chain reaction (PCR) product of the SHV-12 ESBL gene was 865 bp. The PCR product was purified using a PCR purification kit [Takara Biotechnology (Dalian) Co., Ltd.] and cloned into the pMDTM 18-T vector. Recombinant plasmids were transformed into *E. coli* DH5α using T-A cloning; the transformants were selected using PCR and were then subjected to DNA sequencing. The PCR cycle conditions were as follows: Initial denaturation at 95˚C for 5 min; denaturation at 95˚C for 1 min, annealing at 52˚C for 30 min and extension at 72˚C for 1 min, repeated for 30 cycles; final extension at 72˚C for 5 min. The resulting sequences were aligned with available GenBank data using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

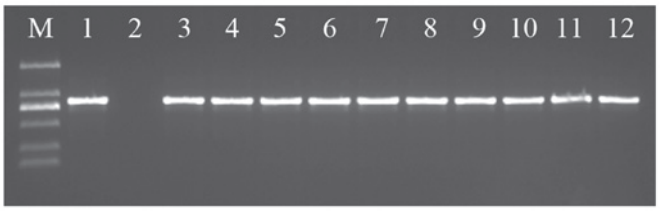

Figure 1. Polymerase chain reaction amplification of the SHV-12 extended-spectrum β-lactamase gene in experimental isolates. M, DNA ladder (from top to bottom: 2,000, 1,000, 750, 500, 250 and 100 bp); 1, positive control; 2, negative control; 3-12, experimental isolates.

Genotyping of enterobacteria. Enterobacterial repetitive intergenic consensus (ERIC)-PCR was used to genotype the clinical isolates. ERIC1R (5'-ATGTAAGCTCCTGGG GATTCAC-3') and ERIC2R (5'-AAGTAAGTGACTGGG GTGAGCG-3') primers that were complementary to ERIC sequences of *E. cloacae* genomic DNA were used for ERIC-PCR, which was performed as described by Stumpf *et al* (15).

Conjugation assay. Mueller-Hinton agar (National Institute for Food and Drug Control) containing rifampicin (200 μg/ml) and ceftriaxone (5 μg/ml) was used to select the *E. cloacae*. Rifampicin-resistant *E. coli* C600 ($LacZ^-$ Nal^r F^- Rif^r) was used as the recipient strain, and the ungrown C600 was used as the donor strain. *E. coli* transconjugants were selected on Mueller-Hinton agar plates containing rifampicin (200 μg/ml) and ceftriaxone (5 mg/l), and bacterial colonies with rifampicin and ceftriaxone-resistance were inoculated onto Eosin-Methylene blue agar plates (containing the same concentration of rifampicin and ceftriaxone). Colorless bacterial colonies on Eosin-Methylene blue agar plates were considered as transconjugants. PCR amplification of the SHV-12 ESBL gene was performed using plasmid DNA of the transconjugant as a template and C600 donor strain as a negative control.

Statistical analysis. An RxC contingency table for the χ^2 test, 2x2 contingency table for the χ^2 test and Fisher's exact probability were used to assess whether statistical difference could be found in the antibiotic resistance rates between the positive and negative isolates, as well as among the three gene locations for positive isolates.

SHV-12 ESBL-positive and -negative isolates were designated as groups 1 and 2, respectively. Group 3 consisted of *E. cloacae* Types A, B and C, as typed by ERIC-PCR, while group 4 consisted of other types. Group 5 consisted of isolates that tested positive in the conjugation assay, while group 6 consisted of isolates that tested negative. For the comparison between groups 1 and 2, and groups 3 and 4, variables in these groups with P-values <0.1 in univariate analyses were separately entered into a multiple logistic regression model to identify the independent risk for *E. cloacae* carrying SHV-12 ESBLs and vertical spread, respectively. Yates' χ^2

Table I. Antibiotic resistance and SHV-12 ESBL.

Antibiotic	Resistant isolates, n (%)		χ^2	P-value	Total resistance rate, %
	SHV-12-positive	SHV-12-negative			
AM	58 (100)	42 (100)			100
CEF	58 (100)	42 (100)			100
CXM	43 (74)	30 (71)	0.09	>0.05	73
CAZ	39 (67)	20 (48)	3.88	<0.05	59
FEP	18 (31)	10 (24)	1.59	>0.05	28
FOX	55 (95)	27 (64)	15.40	<0.05	83
TZP	8 (14)	3 (7)	1.10	>0.05	11
SCF	27 (47)	12 (29)	3.31	>0.05	39
AZT	37 (57)	14 (24)	9.04	<0.05	51
IMP	0 (0)	0 (0)	0.00		0
CIP	22 (38)	8 (19)	4.14	<0.05	30
OFL	24 (41)	18 (43)	0.02	>0.05	42
AMK	9 (16)	3 (7)	1.62	>0.05	12
GEN	24 (41)	9 (21)	4.39	<0.05	33
SXT	31 (53)	11 (26)	7.43	<0.05	42

SHV-12-positive, n=58; SHV-12-negative, n=42. ESBL, extended-spectrum β-lactamase; AM, ampicillin; CEF, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; TZP, tazobactam + piperacillin; SCF, sulbactam + cefoperazone; AZT, aztreonam; IMP, imipenem; CIP, ciprofloxacin; OFL, levofloxacin; AMK, amikacin; GEN, gentamycin; SXT, sulfamethoxazole.

test or exact probability was used to analyze the difference between groups 5 and 6. All comparisons were performed using SPSS 17 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference for all tests.

Results and Discussion

Association between the SHV-12 ESBL gene and the resistance of E. cloacae to antibiotics. The SHV-12 ESBL gene was detected in all 58 *E. cloacae* isolates expressing ESBLs (Fig. 1). This percentage is higher than that reported in France (16) and Taiwan (9), but lower than that in Tunisia (17). A total of 34.48% (20 out of 58 isolates) of the SHV-12 ESBL genes were located in the bacterial chromosome, 48.28% (28 out of 58 isolates) in plasmids and 17.24% (10 out of 58 isolates) in both (Tables I and II).

As shown in Table I, SHV-12 ESBL-positive *E. cloacae* isolates demonstrated significant differences in antibiotic resistance against β-lactam antibiotics (CAZ, FOX, AZT), fluoroquinolone antibiotics (CIP), aminoglycoside antibiotics (GEN) and sulfonamides (SXT) compared with SHV-12 ESBL-negative isolates (P<0.05). It is known that ESBL-expressing *E. cloacae* carry resistance against fluoroquinolone antibiotics (18). Additionally the highly prevalent class I integron contains antibiotic resistance genes, mainly for aminoglycoside antibiotics and trimethoprim (19,20). Similar results have been reported in isolates from Korean hospitals; however, SHV-12 ESBL-positive and -negative isolates did not show significant differences in antibiotic resistance to the β-lactam antibiotics (21). Other mechanisms may, therefore, also contribute to β-lactam antibiotic resistance.

The AMK-resistance rate in the SHV-12 ESBL expression strains was found to be 16% in the present study, which is between that in Mexico (25%) and Taiwan (0%) (22,23). The AM resistance rate, however, was 100%, which is higher than that in Mexico (13%) (22). The fact that the antibiotic-resistance rate of *E. cloacae* in the current region is different from that in other areas may derive from the difference in drug usage and disease type.

SHV-12 ESBL gene location affects antibiotic resistance. The present results showed that all three types of gene location of SHV-12 ESBL could be found in the Guangdong region: A total of 34.48% (20 out of 58 isolates) of the SHV-12 ESBL genes were located in the bacterial chromosome, 48.28% (28 out of 58 isolates) in plasmids and 17.24% (10 out of 58 isolates) in both (Table II). The proportion of each type of location of SHV-12 ESBL in *E. cloacae* was similar to that of SHV ESBLs in *E. coli* in India (24), but was different from the chromosomal location of SHV-12 ESBL in *Klebsiella pneumoniae* in Egypt (6), indicating that the gene location of SHV-12 ESBL may be distinct in different regions or different strains.

In the present study, it was found that the three types of gene locations exhibited significant differences in antibiotic resistance rates for CXM, CAZ, SCF, OFL, GEN and SXT (Table II). The resistance rate for CXM, for example, was higher in the *E. cloacae* isolates carrying the chromosomal SHV-12 ESBL gene (100%) than that in isolates carrying both plasmid and chromosomal SHV-12 ESBL genes (60%).

Table II. Antibiotic resistance and gene location of SHV-12 ESBL.

Gene location (no. of isolates)	No. of resistant isolates												
	AM	CEF	CXM	CAZ	CPM	FOX	TZP	SCF	AZT	IMP	CIP	OFL	AMK	GEN	SXT
Chromosome (20)	20	20	20	7	8	18	2	7	12	0	5	4	1	4	15
Plasmid (28)	28	28	17[a]	22[a]	5	27	4	11	18	0	12	13	7	10	10[a]
Chromosome + plasmid (10)	10	10	6[a]	10[a]	5	10	2	9[a,b]	7	0	5	7[a]	1	8[a,b]	6

[a]P<0.05, compared with chromosome; [b]P<0.05, compared with plasmid. ESBL, extended-spectrum β-lactamase; AM, ampicillin; CEF, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; TZP, tazobactam + piperacillin; SCF, sulbactam + cefoperazone; AZT, aztreonam; IMP, imipenem; CIP, ciprofloxacin; OFL, levofloxacin; AMK, amikacin; GEN, gentamycin; SXT, sulfamethoxazole.

The opposite was true for CAZ; however, certain antibiotics, such as TZP, were unaffected by the gene location of SHV-12 ESBL, indicating that the mechanism of antibiotic resistance in *E. cloacae* is complicated and may be compounded by both internal and external factors.

EPIC-PCR typing. A total of 100 *E. cloacae* isolates were divided into 82 different chromosomal gene types via ERIC-PCR. The isolates were divided into three groups: A, B and C. Type A (9 isolates) and type B (6 isolates) all tested positive for the SHV-12 ESBL gene. Type C isolates (of which there were 6) tested negative for the SHV-12 ESBL gene. The remaining 79 isolates belonged to an orphan clone (Figs. 2 and 3). Of type A, 6 isolates carried chromosome-coded SHV-12 ESBL, while 3 isolates carried both chromosomal and plasmid-coded SHV-12 ESBL. Of type B, 4 isolates carried the chromosomal-coded gene, 1 carried the plasmid-coded gene and 1 carried both. The differential location of the SHV-12 ESBL gene within and between different bacterial gene types indicated that *E. cloacae* carrying SHV-12 ESBL could be transmitted via both vertical and horizontal transmission.

The ERIC-PCR result showed that *E. cloacae* of the same gene type could be detected throughout different clinical departments, suggesting an inter-departmental spread of *E. cloacae* (data not shown). Vertical spread, even outbreaks of *E. cloacae* carrying SHV-12 ESBLs, has also been reported in various regions of the world (7,17,25). In this study, type C *E. cloacae* carrying no SHV-12 ESBL were also found to spread between different clinical departments, implying that other factors could also contribute to the spreading of antibiotic-resistant strains in hospital.

Transconjugation experiment. A total of 31 *E. cloacae* isolates carrying rifampicin (200 μg/ml) plus ceftriaxone (5 mg/l) resistance were selected for the transconjugation experiment. Of these isolates 52%, carried the SHV-12 ESBL gene. In 94% of the cases, antibiotic resistance of *E. cloacae* could be transferred into *E. coli,* which indicated that plasmid-mediated resistance was common in *E. cloacae* and that antibiotic resistance genes could be easily spread via horizontal transmission. The positive rate of transconjugation in the Taiwan region, however, has been shown to be only 70% (26). In 84% (26/31) of the transconjugation cases of the present study, only partial antibiotic resistance genes could be transferred, demonstrating that certain antibiotic resistance genes could not be transferred horizontally. It was also reported that antibiotic resistance genes in *Aeromonas* spp. could not be transferred horizontally. In 10% of the cases in the present study, *E. coli* acquired complete resistance from *E. cloacae,* which indicated that the antibiotic resistance plasmid in *E. cloacae* could mediate complete antibiotic resistance (Table III).

In 64% of the cases, the SHV-12 ESBL gene could be transconjugated successfully from *E. cloacae* to *E. coli,* which meant that horizontal transmission was a major method for the spread of SHV-12. A high prevalence of plasmid-encoded SHV-12 ESBL producers has been observed in the Sichuan province of China (27) and Tunisia (16).

Through the Yates' χ^2 or Fisher's exact probability test, it was found that the factors that improved transconjugation

Table III. Conjugation assays.

SHV-12 location type (n)	Positive test, n (%)	Transconjugants, n (%)		
		SHV-12 positive	Acquired partial resistance	Acquired complete resistance
Chromosome (6)	5 (83)	0 (0)	5 (83)	1 (17)
Plasmid (9)	9 (100)	6 (67)	7 (78)	2 (22)
Chromosome and plasmid (2)	2 (100)	1 (50)	2 (100)	0 (0)
SHV-12 negative (14)	13 (93)	0 (0)	12 (92)	1 (8)
Total (31)	29 (94)	7 (23)	26 (84)	3 (10)

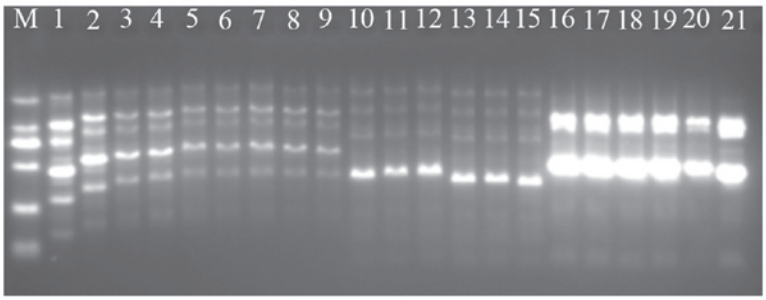

Figure 2. Enterobacterial repetitive intergenic consensus-polymerase chain reaction for type A, B and C isolates. M, DNA ladder (from top to bottom: 2,000, 1,000, 750, 500, 250 and 100 bp); 1-9, type A isolates; 10-15, type B isolates; 16-21, type C isolates.

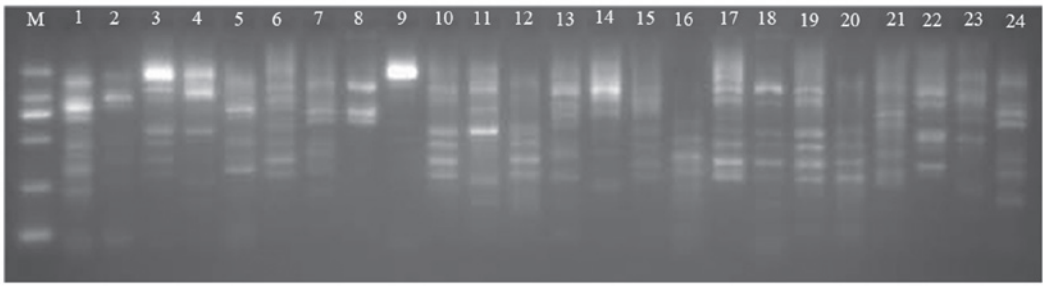

Figure 3. Enterobacterial repetitive intergenic consensus-polymerase chain reaction for different types of chromosomal DNA. The isolates with identical patterns of the same location of bands or same number of bands with different density were considered to be the same genotype; the isolates with differences in 1-2 bands were considered to be closely a related genotype; the isolates with differences in >2 bands were considered to be a different genotype. M, DNA ladder (from top to bottom: 2,000, 1,000, 750, 500, 250 and 100 bp); 1-24, experimental isolates.

rates in patients were male gender, use of antibiotics in the last 30 days, hospital stays lasting >14 days and Charlson comorbidity index (CCI) >2 ($P<0.05$). This result also indicated that these factors could affect the horizontal transmission of antibiotic resistance in clinical niches.

Association between external factors and E. cloacae antibiotic resistance. The *E. cloacae* strains were isolated from 7 types of samples collected from 18 clinical departments (Table IV), which meant that *E. cloacae* could cause various infections. In the ICU and respiratory department, *E. cloacae* had the highest (30%) and second highest (11%) positive rates, respectively. In Taiwan, the ICU is also the most common department in which *E. cloacae* strains have been isolated (9). In the region analyzed in the present study, the majority of the *E. cloacae* strains were from the sputum; this is similar to the situation in Tunisia and southern Brazil (17), but different from France and Taiwan, where the urinary tract is the most common infection site for *E. cloacae* (28).

Respiratory tract infection caused by *E. cloacae* occurs in different age groups. Patients aged ≥60 years had the highest *E. cloacae* infection rate (54%), and male patients were more likely to be infected by *E. cloacae* than female patients (62 male vs. 38 female patients; male to female ratio, 1.63:1). A similar effect of age and gender on *E. cloacae* infection rate has also been reported in other regions of the world (16).

In total, >50% of the patients infected with *E. cloacae* were undergoing invasive treatment, had underlying diseases and/or complications, had been taking antibiotics for >30 days, were hospitalized for >14 days or had a CCI

Table IV. External factors and antibiotic resistance.

Factor	Isolates, n (%)					
	Group 1 (n=58)	Group 2 (n=42)	Group 3 (n=21)	Group 4 (n=79)	Group 5 (n=72)	Group 6 (n=3)
Source of isolates						
Sputum	28 (48)	21 (50)	16 (76)	33 (42)	35 (78)	3 (100)
Urine	10 (17)	12 (29)	3 (14)	19 (24)	13 (18)	0 (0)
Other sources[1]	20 (35)	9 (21)	2 (10)	27 (34)[b]	24 (33)	0 (0)
Clinical department						
Intensive Care Unit	27 (47)	3 (7)[a]	11 (52)	19 (24)[b]	27 (38)	0 (0)
Respiratory Medicine	6 (10)	5 (12)	3 (14)	8 (10)	8 (11)	0 (0)
Other departments[2]	25 (43)	34 (81)[a]	7 (33)	52 (66)[b]	37 (51)	3 (100)
Age in years						
1-14	7 (12)	2 (5)	0 (0)	9 (11)	7 (10)	1 (33)
15-59	24 (41)	13 (31)	5 (24)	32 (41)	28 (39)	2 (33)
≥60	27 (47)	27 (64)	16 (76)	38 (48)[b]	37 (51)	0 (0)
Gender						
Male	40 (69)	22 (52)	11 (52)	51 (65)	50 (69)	0 (0)
Female	18 (31)	20 (48)	10 (48)	28 (35)	22 (31)	3 (100)
Other factors						
Invasive procedure	44 (76)	20 (48)[a]	16 (76)	48 (61)	49 (68)	2 (67)
Presence of any underlying illnesses and/or comorbidities	56 (97)	34 (81)[a]	21 (100)	69 (87)	64 (89)	2 (67)
Use of antibiotics in the last 30 days	53 (91)	39 (93)	21 (100)	71 (90)	67 (93)	0 (0)[c]
Hospitalization >14 days	53 (91)	31 (74)[a]	21 (100)	63 (80)	60 (83)	0 (0)[c]
CCI >2	51 (88)	29 (69)[a]	19 (90)	61 (77)	60 (83)	0 (0)[c]
Hospital patients	57 (98)	42 (100)	21 (100)	77 (97)	71 (99)	3 (100)
Mortality	5 (9)	0 (0)	4 (19)	1 (5)	4 (6)	0 (0)

[a]P<0.05, compared with group 1; [b]P<0.05, compared with group 2; [c]P<0.05, compared with group 5. [1]Other sources includes blood (n=13), exudates (n=5), pus (n=4), bile (n=4) and pleural fluid (n=3); [2]Other departments includes the departments of Internal Hematology (n=9), Neurology (n=6), Urology (n=5), Pediatrics (n=5), Cardiothoracic Surgery (n=5), Urological Surgery (n=4), Orthopedic Surgery (n=4), Hepatobiliary Surgery (n=4), Neurosurgery (n=3), Geriatrics (n=3), Plastic Surgery (n=2), Pediatric Surgery (n=1) and Clinical Infectious Diseases (n=1), as well as the Coronary Care Unit (n=3) and section for outpatients (n=1). CCI, Charlson comorbidity index.

>2, which further illustrated that infection with *E. cloacae* is closely associated with external factors; this is consistent with other investigations (10-12).

Notably, antibiotic abuse has been considered an essential reason for antibiotic resistance and the wide spread of antibiotic-resistant strains; however, no significant effect of antibiotic usage for >30 days was found in groups 1-4. A possible reason could be the 3-year application of antibiotic usage policy in Guangdong, where the samples were collected. In this study, a higher positive rate of ESBLs was found compared with other regions of China. One possible explanation is that the antibiotic resistance gene is preserved in the strain for long periods of time and spread once it is captured by mobile genetic elements, such as integrons (29).

As analyzed by multiple logistic regressions, the risk factors for infection by SHV-12-producing *E. cloacae* were age, clinical department and having an underlying disease or complication (P<0.05). In a previous study, the risk factors for suffering bloodstream infections of *E. cloacae* carrying ESBLs were disease severity, category of healthcare-associated infection and prior use of antibiotics or a ventilator (23). This difference may be associated with the method of drug administration used by the doctors and the sample source.

E. cloacae isolated from the bile had the highest antibiotic resistance rate compared with those isolated from the other sources (data not shown). Although the positive rate for *E. cloacae* was low in the pus, secretions, bile and chest water, the majority of the *E. cloacae* detected were positive for SHV-12 ESBL, which is noteworthy for clinicians.

E. cloacae isolated from ICU samples had the highest positive rate for SHV-12 ESBLs. This may have been due to the fact that those patients in the ICU had an increased incidence of underlying diseases or complications. Additionally, ICU patients often have undergone prior stays in other departments or have used broad-spectrum antibiotics, which may increase the possibility of acquiring SHV-12 ESBLs for *E. cloacae* (28).

Multiple logistic regression analysis results showed that the risk factors for vertical transmission were infection site, age and clinical department. The horizontal spread of SHV-12 ESBLs was the most common between *E. cloacae* strains separated from the sputum (76%), the elderly (76%) and ICU patients (52%), which is consistent with the most common sample type, clinical department and patient age in this region. Horizontal transmission of *E. cloacae* was reported in an ICU in Croatia, and was common in respiratory tract infections and the elderly (30).

In conclusion, the expression pattern, gene location and transmission pathways of SHV-12 ESBL and other external factors have a close correlation with the antibiotic resistance of *E. cloacae*. To prevent the wide spread of SHV-12 ESBL, as well as outbreaks of multi-drug-resistant strains, we propose that more attention be given to the clinical investigation of SHV-12 ESBL-expressing *E. cloacae* to better monitor its spread and provide evidence for the proper usage of antibiotics.

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