Infection and Drug Resistance

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ORIGINAL RESEARCH

Phenotypic and genotypic characterization of multi-drug-resistant *Escherichia coli* isolates harboring bla$_{CTX-M}$ group extended-spectrum β-lactamases recovered from pediatric patients in Shenzhen, southern China

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Aims and Objectives: The emergence and spread of extended-spectrum β-lactamases (ESBLs) particularly CTX-M producing multi-drug-resistant (MDR) *Escherichia coli* (*E. coli*) is one of the greatest challenges for community health globally. The study investigated the phenotypic and genotypic characteristics of ESBLs-producing *E. coli* recovered from pediatric patients from Shenzhen Children's Hospital, China.

Materials and methods: Present study, a total of 2,670 isolates of *E. coli* were collected from Shenzhen Children's Hospital, China of which 950 were ESBLs producer. ESBLs production was confirmed by using the combination disc diffusion method, and antimicrobial susceptibility test was detected. In addition, β-lactamase-producing genes and co-existence of carbapenem/colistin resistance genes were determined by PCR assay and sequencing. The diversity and phylogenetic relationship were determined by multi-locus sequence typing method.

Results: Thirty-five percent (n=950) prevalence of ESBLs-producing *E. coli* we reported in Shenzhen, China of which 50 ESBLs producing *E. coli* were randomly selected for a further characterization. All 50 ESBLs- producing *E. coli* isolates revealed MDR phenotype and 100% were resistant to Ampicillin/sulbactam, Ampicillin, Cefazolin, and Ceftriaxone. All 50 ESBLs producers harbored at least one type of β-lactamase gene particular *bla*$_{CTX-M}$. The PCR and sequencing revealed the most common CTX-M subtype was *bla*$_{CTX-M-15}$ (n=18), followed by *bla*$_{CTX-M-14}$ (n=16), *bla*$_{CTX-M-90}$ (n=9), *bla*$_{CTX-M-55}$ (n=3), *bla*$_{CTX-M-27}$, *bla*$_{CTX-M-101}$, and *bla*$_{CTX-M-211}$ each (n=1). Co-existence of *bla*$_{CTX-M}$ with *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{GES}$, and *bla*$_{VEB}$ was detected in few isolates. Among identified sequence types, ST131 (12%) was more dominant in ESBLs-producing *E. coli*. Phylogenetic group A was the most prominent group among the ESBLs-producing *E. coli* based on multiplex PCR.

Conclusion: Our study shows the prevalence of *bla*$_{CTX-M}$ gene in ESBLs-producing *E. coli* in pediatric patients in Shenzhen, China. We highlight the importance to monitor the emergence and trends of ESBLs-producing isolates in a pediatric healthcare setting.

Keywords: Antimicrobial resistance, molecular characterization, MLST, ESBLs, *Escherichia coli*

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Introduction

The swift emergence of antibiotic-resistant *Enterobacteriaceae* family is the major cause of hospital admission and associated morbidity and mortality in children.[1] *Enterobacteriaceae*,

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predominantly Escherichia *coli* is a significant opportunistic pathogen causing infections in hospitals and serves as a key cause of urinary tract infections, gastrointestinal tract infections, bloodstream infection, and meningitis in humans.[2,3] *E. coli* is a major reservoir of the Extended-spectrum β-lactamases (ESBLs) encoding genes.[4] ESBLs are able to hydrolyze the modern β-lactam antibiotics including third- generation Cephalosporin. A total of 350 dissimilar ESBLs variant has been identified till date. which are divided into nine separate families based on the amino acid sequences such as TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA, and OXA.[5,6] Among them, TEM, SHV, CTX-M, and OXA are the major variants reported globally. Particularly *bla*$_{CTX-M}$ has been increased rapidly and is now widely found in clinical isolates of *E. coli* across the world.[7,8] Some studies have demonstrated that ESBLs-producing *E. coli* has become an epidemic in China.[9–11] However, all these studies focused on food, environment, and adult clinical cases. So far, little is known about the epidemiology of ESBLs-producing *E. coli* in pediatric patients from southern China. Moreover, it is critical to provide up-to-date resistance pattern which guides the treatment decision in southern China. Therefore, this study was aimed to investigate the phenotypic and genotypic characterization of ESBLs-producing *E. coli* recovered from pediatric patients in Shenzhen Children's Hospital, China.

Materials and methods

Bacterial isolation and identification

A total of 2,670 unique clinical *E. coli* isolates were collected (one isolate recovered from one child) between January 2014 and December 2015 from Shenzhen Children's Hospital, China. This hospital is a major children hospital in the southern area of China. Among the 2,670 *E. coli* isolates, 950 (35%) were confirmed as ESBLs-producing *E. coli* by VITEK2 compact system (Ref. No. 27530/275660) of which 50 were randomly selected for molecular analysis. Among the 50 ESBLs- producing *E. coli*. isolates 32 (64%) were from male and 18 (36%) were from female, patients age ranges from 1 month to 12 years. The clinical isolation site for specimens was as follows, urine *n*=16, sputum *n*=16, pus *n*=12, catheter-associated *n*=3, blood *n*=2 and cerebral spinal fluid *n*=1(S-1).

Phenotypic detection of ESBLs production

The combination disc test was done for phenotypic detection of ESBLs production. The test was performed by using the disc of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid. Control strain, which was selected from the characterized strain collection of our laboratory while ATCC25922 used as a negative control strain. The ESBLs production result was analyzed according to the Clinical and Laboratory Standards Institute (CLSI) guideline.[12]

Antimicrobial susceptibility test

Antimicrobial susceptibility was performed by VITEK@2 compact system (Biomerieux-Ref. No. 27530/275660) method for 18 antimicrobial agents, namely, Ampicillin/ Sulbactam, Piperacillin, Ertapenem, Amikacin, Levofloxacin, Nitrofurantoin, Ampicillin, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Imipenem, Cefotetan, Tobramycin, Gentamicin, and Ciprofloxacin. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guideline.[12]

Detection of β-lactamase and associated genes

The standard PCR was performed to detect the presence of ESBLs encoding genes: *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$(variant), *bla*$_{GES}$, *bla*$_{CARB}$, *bla*$_{PER}$, *bla*$_{VEB}$, and *bla*$_{OXA}$ using specific primers previously described.[13] In addition, carbapenemase genes (*bla*$_{KPC}$ and *bla*$_{NDM-1}$) and colistin resistance *mcr-1* were determined in ESBLs-producing *E. coli* by PCR assay and sequencing. The specific primers were used as described in our previous study.[14] The purified PCR products were sequenced commercially (Sangon Biotech-Shanghai, China). DNA Sequences were analyzed by NCBI-BLAST program.

Multi-locus sequence typing (MLST)

The sequences types (STs) were determined for ESBLs-producing *E. coli* isolates by MLST. PCR assay was performed to amplify internal portions of seven housekeeping genes of *E. coli* (*adk, fumC, gyrB, icd, mdh, purA*, and *recA)* with specific primers.[15] Amplified products were sequenced commercially (Sangon Biotech-Shanghai, China). The allelic type and sequences type for all ESBLs-producing *E. coli* were determined with achtman scheme available at *http://mlst.warwick.ac.uk/mlst/dbs/Ecoli*.

Phylogenetic group detection

Major phylogenetic group of all ESBLs-producing *E. coli* isolates was determined by multiplex PCR assays, using the combination of three DNA markers genes (*chuA, yjaA*, and *TspE4.C2*) as described by Clermont et al[16].

Plasmid transferability

Conjugation experiments were performed to analyze the horizontal gene transfer of bla_{CTX-M} for ESBLs-producing *E. coli* isolates by using streptomycin-resistant *E. coli* C_{600} as the recipient strain. We used liquid mating assay as described earlier.[17] Transconjugants were selected on Luria Bertani agar containing streptomycin 2,000 (μg/mL) and cefotaxime (32 μg/mL). Transconjugants were further tested for ESBLs production and the existence of bla_{CTX-M} genes by PCR.

PCR-based replicon typing

PCR-based replicon typing was performed for both plasmids from parental and transconjugant isolates. The Inc (Incompatibility) groups were determined by using specific primer introduced by Carattoli et al, in 2005.[18]

Results

ESBLs production and antimicrobial susceptibility

A total of *n*=950 shown ESBLs production by VITEK2 compact system (Ref. No. 27530/275660) of which randomly selected 50 *E. coli* further confirmed as ESBLs-producing *E. coli* by combination disc test. The result was analyzed according to the CLSI guideline. Antimicrobial susceptibility tests reflected that all of the randomly selected 50 ESBLs -producing *E. coli* isolates 100% were resistant to Ampicillin/sulbactam, Ceftriaxone, Cefazolin, and Ampicillin while Aztreonam (50%), Trimethoprim (60%), Gentamycin (38%), Ciprofloxacin (32%), Cefepime (30%), Cefotaxime (28%), Tobramycin (14%), and Nitrofurantoin (3,8%). However, all of the isolates were susceptible to Piperacillin, Ertapenem, Amikacin, Imipenem, and Cefotetan. The antimicrobial phylogram was analyzed by *Bio-numeric* software (Figure 1). The antimicrobial results indicate that all of 50 ESBLs-producing *E. coli* are resistant to two more than two class of antibiotics so-called as multi-drug resistant *E. coli*. According to the results obtained, resistance to Aztreonam and Cefepime showed a significant difference for the time period during January 2014–July 2014 and July 2014–December 2015.

Molecular analysis of drug resistance genes

All of the 50 ESBLs-producing *E. coli* isolates were carrying bla_{CTX-M} genes, with the most common being $bla_{CTX-M-15}$ (18/50, 36%), followed by $bla_{CTX-M-14}$ (16/50, 32%) and $bla_{CTX-M-90}$ (9/50, 18%), $bla_{CTX-M-55}$ (3/50, 6%) $bla_{CTX-M-101,\ 211,\ 27,\ 109}$ (1/50, 2%). Additionally, co-existence of other β lactamase genes was detected, bla_{TEM} (10/50,20%) followed by bla_{SHV} (8/50,16%), bla_{GES} (5/50,10%), bla_{CARB} (1/50,2%) (Table 1). The bla_{PER}, bla_{VAB}, and bla_{OXA} group genes were not detected in this study. It was noteworthy that all $bla_{CTX-M-14}$ gene carrying ESBLs-producing *E. coli* isolates were resistant to Ciprofloxacin. There was no significant difference in the prevalence of bla_{CTX-M} genes among the ESBLs-producing *E. coli* isolated from the different isolation sites or even samples. Moreover, carbapenemase-producing genes were detected, including bla_{NDM-1} (14/50, 28%), bla_{KPC} (5/50, 10%), and most recently discovered colistin resistance *mcr-1* (2/50,10%) (Table 1).

Multi-locus sequences typing and phylogenetic grouping

The extensive diversity of MLST was recorded from ESBLs-producing *E. coli* isolates, with a total of 30 different STs of which, ST131 (12%) was highly prevalent in Shenzhen (Table 1). *E. coli* ST95 clonal complex (CC) was the major complex observed among all studied isolates. The ST95CC has been usually observed from urine, sputum, and pus samples. All ST131CC isolates were recovered from general surgery wards, these results indicate that ST131CC *E. coli* was protuberant in the general surgery wards and key transporter for the $bla_{CTM-M-14}$ gene (Table 1). Our particular concern is that $bla_{CTM-M-15}$ gene was reported in different 15 STs in Shenzhen Children's Hospital. This observation suggested that ESBLs-producing *E. coli* isolates carrying $bla_{CTM-M-15}$ gene spread in the Shenzhen region and are now widespread in Southern China. All ESBLs-producing *E. coli* isolates were classified into four phylogenetic groups, namely, A, B1, D, and B2. The results revealed that majority of isolates belonged to group A (54%), along with a substantial proportion for groups B1 (22%), D (8%), and B2 (6%).

Plasmid profiling

The successful transconjugants were selected from Luria Bertani agar containing streptomycin 2,000 (μg/mL) and cefotaxime (32 μg/mL). We observed that IncFIA(*n*=14), IncHI2 (*n*=10), IncFIB (*n*=10), IncFIIS(*n*=3), IncFIC (*n*=2), and IncFH1(*n*=2) "Inc" group plasmids were responsible for the horizontal gene transformation of *bla*CTX-M genes **(S 2)**. Nine transconjugants isolates were not shown any "Inc" group.

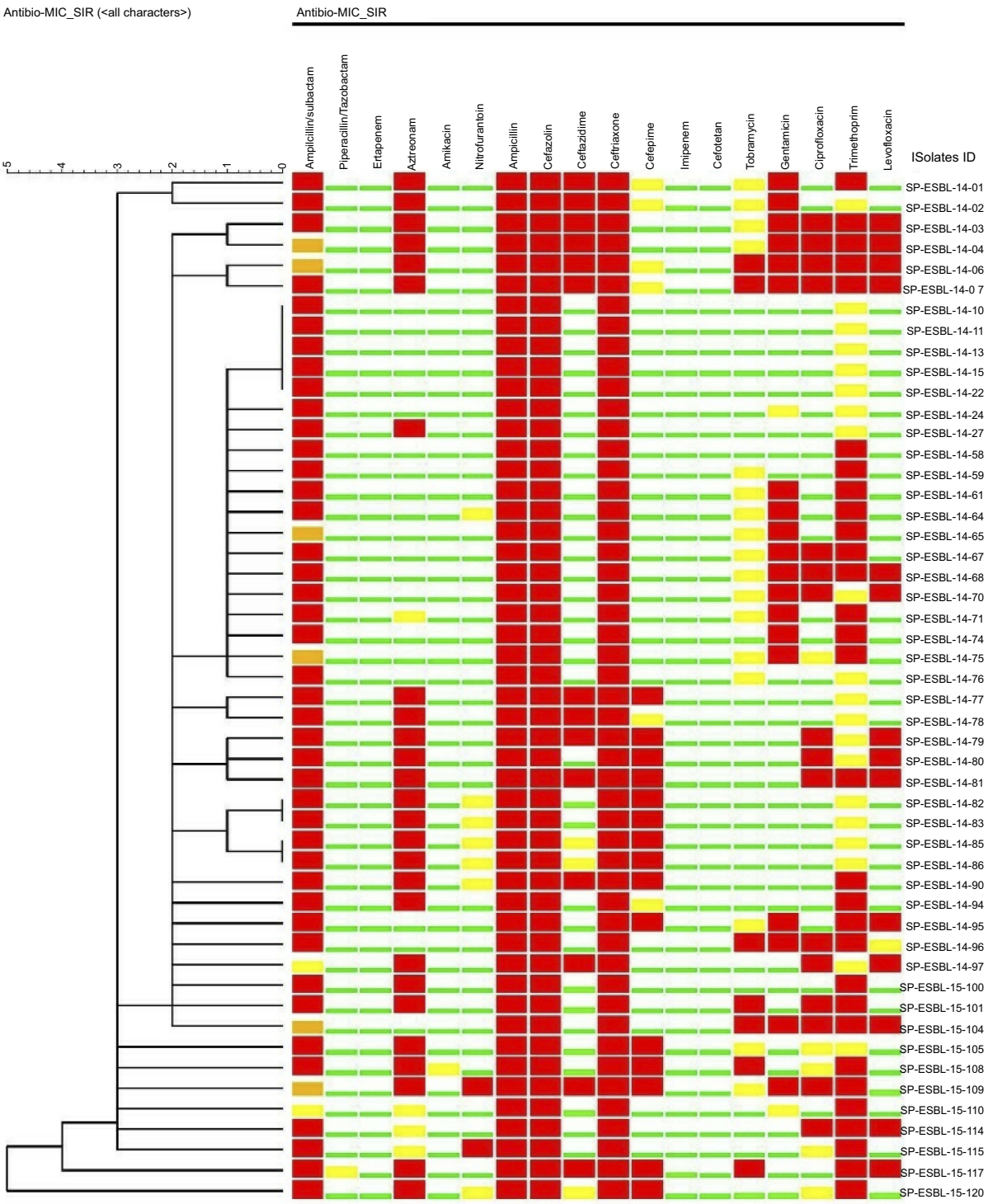

Figure 1 Antimicrobial susceptibility pattern and phylogram of ESBLs producing *E.coli*, Red color indicates resistant; yellow color indicates intermediate resistant; Green color indicates sensitive.

Abbreviations: *E.coli*, *Escherichia coli;* ESBL, extended-spectrum β-lactamases.

Discussion

The incidence of CTX-M-type ESBLs among clinical isolates especially *E. coli* has noticeably increased in the earlier several years.[19] To the best of our knowledge, this is the first study from southern China to precisely demonstrate the prevalence of CTXM-type ESBLs and antimicrobial susceptibility pattern of *E. coli* which were isolated from pediatric infectious cases. The prevalence of ESBLs-producing *E. coli* in our study was 35% which was lower than the across China-northwest (71.7%), southwest (61.1%), north

Table 1 β-lactamase encoding gene analysis and STs of ESBLs-producing *E. coli*

Isolates	ESBLs genes					Carbapenemase gene		MCR-1 gene	STs	CC
	bla$_{CTX-M}$	*bla*$_{SHV}$	*bla*$_{CARB}$	*bla*$_{GES}$	*bla*$_{TEM}$	*bla*$_{KPC}$	*bla*$_{NDM-1}$	*MCR-1 gene*		
SP-ESBL-14-01	CTX-M-14	+	–		+	–	–		ST451	None
SP-ESBL-14-02	CTX-M-15		–		–	–	–	–	ST597	ST69CPLX
SP-ESBL-14-03	CTX-M-14	+	–		–	–	–	–	ST1170	None
SP-ESBL-14-04	CTX-M-211	–	–		–	–		-	ST159	None
SP-ESBL-14-06	CTX-M-14	–	–		–	-	+	-	ST95	ST95CPLX
SP-ESBL-14-07	CTX-M-15	–	–		–	-	-	-	ST205	ST205CPLX
SP-ESBL-14-10	CTX-M-15	–	–		–	-	+		ST1177	ST38CPLX
SP-ESBL-14-11	CTX-M-14	–	–	+	–	-	-	-	ST531	ST95CPLX
SP-ESBL-14-13	CTX-M-15	–	–		–	-	-	-	ST648	ST648CPLX
SP-ESBL-14-15	CTM-M-15	+	–		+	-	+	+	ST159	None
SP-ESBL-14-22	CTX-M-15	–	–		+	-	-	-	ST131	ST131CPLX
SP-ESBL-14-24	CTX-M-14	–	–	+	+	-	+	-	ST131	ST131CPLX
SP-ESBL-14-27	CTX-M-15	+	–		–	-	+		ST915	None
SP-ESBL-14-58	CTX-M-14	+	-		-	+	+	-	ST701	ST131CPLX
SP-ESBL-14-59	CTX-M-15	+	-		-	+	+	-	ST1461	ST131CPLX
SP-ESBL-14-61	CTX-M-15	-	-		-	-	-	-	ST95	ST95CPLX
SP-ESBL-14-64	CTX-M-109	-	-		-	-	-	-	N/D	N/D
SP-ESBL-14-65	CTX-M-90	-	-		-	-	-	+	ST648	ST648CPLX
SP-ESBL-14-67	CTX-M-101	+	-		-	-	+	-	ST131	ST131CPLX
SP-ESBL-14-68	CTX-M-14	-	-		-	-	+	-	ST106	ST69CPLX
SP-ESBL-14-70	CTX-M-14	-	-		+	-	+	-	ST131	ST131CPLX
SP-ESBL-14-71	CTX-M-14	-	-		-	-	-	-	ST38	ST38CPLX
SP-ESBL-14-74	CTX-M-15	-	-		-	+	+	-	ST106	ST69CPLX
SP-ESBL-14-75	CTX-M-15	-	+		+	-	+	-	ST117	None
SP-ESBL-14-76	CTX-M-14	-	-		-	-	-	-	ST443	ST205CPLX
SP-ESBL-14-77	CTX-M-90	-	-		-	-	-	-	ST648	ST648CPLX
SP-ESBL-14-78	CTX-M-15	-	-		-	-	-	-	ST495	None
SP-ESBL-14-79	CTX-M-14	-	-		+	-	-	-	ST131	ST131CPLX
SP-ESBL-14-80	CTX-M-55	-	-		-	-	-	-	ST595	None
SP-ESBL-14-81	CTX-M-14	-	-		-	-	+	-	ST127	None
SP-ESBL-14-82	CTX-M-90	-	-		-	-	-	-	ST12	ST12CPLX
SP-ESBL-14-83	CTX-M-15	-	-		-	-	-	-	ST95	ST95CPLX
SP-ESBL-14-85	CTX-M-14	-	-		-	-	-	-	ST12	ST12CPLX
SP-ESBL-14-86	CTX-M-15	-	-		-	-	-	-	ST95	ST95CPLX
SP-ESBL-14-90	CTX-M-14	-	-		-	-	+	-	ST131	ST131CPLX
SP-ESBL-14-94	CTX-M-90	-	-		+	-	-	-	ST444	ST446CPLX
SP-ESBL-14-95	CTX-M-27	-	-		+	-	+	-	ST1416	ST14CPLX
SP-ESBL-14-96	CTX-M-90	-	-		-	-	-	-	ST439	ST446CPLX
SP-ESBL-14-97	CTX-M-90	-	-		-	-	-	-	ST140	ST95CPLX
SP-ESBL-15-100	CTX-M-90	-	-	+	-	-	-	-	ST3201	ST95CPLX
SP-ESBL-15-101	CTX-M-55	-	-		-	-	-	-	ST38	ST38CPLX
SP-ESBL-15-104	CTX-M-15	-	-		-	-	-	-	ST106	ST69CPLX

(*Continued*)

Table 1 (Continued).

Isolates	ESBLs genes					Carbapenemase gene		MCR-1 gene	STs	CC
	bla$_{CTX-M}$	*bla*$_{SHV}$	*bla*$_{CARB}$	*bla*$_{GES}$	*bla*$_{TEM}$	*bla*$_{KPC}$	*bla*$_{NDM-1}$	*MCR-1 gene*		
SP-ESBL-15-105	CTX-M-90	-	-		-	-	-	-	ST140	ST95CPLX
SP-ESBL-15-108	CTX-M-15	-	-		-	-	-	-	ST398	ST398CPLX
SP-ESBL-15-109	CTX-M-14	-	-		-	-	-	-	ST648	ST648CPLX
SP-ESBL-15-110	CTX-M-15	-	-		-	-	-	-	ST140	ST95CPLX
SP-ESBL-15-114	CTX-M-15	-	-		-	-	-	-	ST320	ST69CPLX
SP-ESBL-15-115	CTX-M-90	-	-		-	-	-	-	ST421	ST95CPLX
SP-ESBL-15-117	CTX-M-14	-	-		-	-	+	-	ST10	ST10CPLX
SP-ESBL-15-120	CTC-M-55	+	-		+		+	-	ST2144	None

Abbreviations: *E.coli*, Escherichia coli; ESBL, extended-spectrum β-lactamases; STs, sequences type; CC, clonal complex; N/D, not detected.

(48.2%), and east (46.86%) reported in bloodstream infection.[20] The high prevalence of ESBLs-producing *E. coli* about 82.6% was reported from Taian, a large city in Shandong province, China. But, the prevalence of ESBLs-producing *E. coli* in Shenzhen, China higher than the other nations, namely, Brazil (12.8%), Chile (23.8%) and Argentina (18.1%).[21] We have a lower prevalence of ESBLs-producing *E. coli* than rest of the country may due to sturdy prevention measures, however, we should pay continual attention to tackle this problem by following informed treatment decisions from past experience.

The antimicrobial susceptibility test data clearly indicate that high resistance rate of ESBLs-producing *E. coli* to Ceftriaxone, Cefazolin and Ampicillin (100%), Aztreonam (50%), Trimethoprim (60%), Gentamycin (38%), and Ciprofloxacin (32%) has raised serious concern and became a challenge for clinicians. Therefore, we suggest avoiding indiscriminate use of antibiotics in medical practice which will certainly lower the opportunities for the emergence of resistance. Our antimicrobial susceptibility results were comparable with another part of China, Taiwan, and Thailand.[20,22,23]

We reported, *bla*$_{CTX-M-15}$ as the most prevalent genotype of ESBLs-producing *E. coli* in Shenzhen followed by *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-90}$, *bla*$_{CTX-M-55}$. *bla*$_{CTX-M-101}$, *bla*$_{CTX-M-211}$, *bla*$_{CTX-M-27}$. This result indicates the diversity of CTX-M genotype of ESBLs-producing *E. coli* in Shenzhen, China. Similar results were reported from across China.[24,25] In our study *bla*$_{CTX-M-55}$ is not detected normally in pediatric patients, which means children may not be in contact with an animal since this genotype is mostly circulated via animal origin *E. coli* isolates.[26] Co-existence of CTX-M group gene and β-lactamase genes includes *bla*$_{TEM}$ (20%) followed by *bla*$_{SHV}$ (16%), *bla*$_{GES}$ (10%), *bla*$_{CARB}$ (2%) were detected in ESBLs-producing *E. coli*. The β-lactamase genes detection results indicate that the β-lactamase genes excluding CTX-M group decreased over the period of time comparable study reported by Jiranun Bubpamala.[23] Jiranun Bubpamala reported that CTX-M group genes continually increasing from 2007 to 2018 but other β-lactamase genes were declined. In addition, co-existence of ESBLs with either carbapenem-resistant genes *bla*$_{NDM-1}$ (28%), *bla*$_{KPC}$ (10%), or most recently discovered colistin-resistant *mcr-1* (10%) raises a concern about the spread of such superbugs in the Shenzhen area. Several reports showed the co-existence of carbapenem resistance genes and *mcr-1* in *E. coli* in China.[27] The 41 ESBLs-producing *E. coli* isolates shown six different Inc plasmid groups which include IncFIA, IncHI2, IncFIB, InFIIS, IncFIC, and InFH1 similar types of plasmid group with associated with *bla*$_{CTX-M}$ was reported in China.[28] Though our study was limited by the isolate numbers and geographic

area however the results sufficiently implicate the need of close monitoring of such superbugs in a clinical setting for this region.

MLST results reflect that ST131 (12%) was the most common ST among the 50 ESBLs-producing *E. coli* isolates. Similarly, some current countrywide studies from tertiary and county hospitals have also shown that ST131 was found in 9.6%, 12.7%, and 13.4% of ESBL-producing *E. coli*, respectively.[8,29,30] In contrast, the percentage of ST131 ESBLs-producing *E. coli* is notably lower in China as compared to European and American regions, according to a community infection study in the US (53%), UK (64%), and Belgium (64%).[31,32]

Conclusion

The study demonstrated that $bla_{\text{CTX-M}}$ gene was dominant in ESBLs-producing *E. coli* at Shenzhen Children's Hospital and was composed of a variety of subtypes. We described the ESBLs-producing *E. coli* has developed an increasing level of resistance to antibiotics. Our study stresses on the necessity of long-term monitoring on ESBLs-producing *E. coli* in hospital environments, especially in Shenzhen Children's Hospital. National programs devoted to the health of children in China need to consider the emerging threat of ESBLs-producing bacteria, and research efforts should be devoted to focus on the molecular characterization of ESBL types as well as additional controlled studies assessing risk factors and possible outcomes for children.

Ethics statement

The present study received approval from the Shenzhen Children's Hospital (Research) ethical committee 2018 (013)

The clinical isolates used in this research were part of routine hospital laboratory procedures. Verbal consent was given by the patient's parent/s or legal guardian/s.

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Disclosure

The authors report no conflicts of interest in this work.

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