The Journal of Antibiotics (2016) 69, 169–172

www.nature.com/ja

NOTE

Increased resistance rate to ceftazidime among blood culture isolates of ESBL-producing *Escherichia coli* in a university-affiliated hospital of China

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The Journal of Antibiotics (2016) **69,** 169–172; doi:10.1038/ja.2015.100; published online 30 September 2015

INTRODUCTION

Blood stream infection (BSI) is a common, expensive and frequently fatal condition that significantly increases morbidity and mortality.[1] *Escherichia coli* (*E. coli*) is a common pathogen isolated from blood culture. Effective antimicrobial treatment for *E. coli* is needed to reduce the mortality of patients with BSI. Cephalosporins and ceftazadine are viable therapeutic options to treat *E. coli*-infected BSI due to their effectiveness and low toxicity profiles. However, there have been increasing reports of resistant *E. coli* pathogen in patients with BSI that is less treatable with antibiotic agents because of the extended-spectrum β-lactamase (ESBL) production.[2,3]

Historically, Temoniera (TEM) type and sulphydryl variable (SHV) type are previously recognized as the main types of ESBL. However, the genotypic and epidemiological studies of ESBL demonstrated that cefotaxime (CTX)-M types have replaced TEM type and SHV type as the most common ESBL in China, with CTX-M-14 type being the predominant type.[4] Noticeably, isolates with some CTX-M types, such as CTX-M-14, were found to be more resistant to cefotaxime than to ceftazidime.[5] However, other CTX-M types (CTX-M-15, CTX-M-16, CTX-M-27 and CTX-M-55), which harbor the Asp240Gly substitution,[6–8] confer higher levels of resistance to ceftazidime than their parental enzymes (CTX-M-3, CTX-M-9 and CTX-M-14).

In 2010, breakpoints for *Enterobacteriaceae* against third generation cephalosporins have been revised in the M100-S20 document by the Clinical Laboratory Standards Institute (CLSI).[9] It is recommended that susceptibility results of *Enterobacteriaceae* for cephalosporins be reported according to the minimum inhibitory concentration (MIC), regardless of whether or not the isolate produces an ESBL. Based on the recommendation by the CLSI, many CTX-M-producing *E. coli* should be reported as susceptible to ceftazidime.

To investigate whether the new ceftazidime breakpoint would affect the percentage of ceftazidime resistance, changes in the antimicrobial agent resistance rate and CTX-M genotypic profile were retrospectively reviewed among blood culture isolates of ESBL-producing *E. coli* (ESBL-EC) from a university-affiliated hospital in China from January 2008 to December 2013.

MATERIALS AND METHODS

Ethics statement

This study was conducted in Weihai Municipal Hospital (affiliated to Dalian Medical University) after obtaining due approval from the institutional ethics committee.

Isolation and identification of *E. coli* from blood culture

Cultured blood specimens were collected from adult patients suspected of having BSI. Briefly, 20 ml of clinical blood specimens were subjected to consecutive routine blood culture bottles (BacT/ALERT FA and BacT/ALERT FN). All bottles were incubated at 37 °C in BacT/ALERT 3D automated continuous monitoring system. Bottles that were identified as positive were removed, and then the samples underwent Gram staining, followed by culturing on Columbia blood agar plates and incubating at 37 °C.

Bacterial isolates were identified and confirmed with *E. coli* by the standard microbiological procedures using API system (BioMérieux, Marcy l'Etoile, France) and VITEK 2 Compact (BioMérieux). In cases where the same strains were repeatedly isolated from the blood of a patient, only the first isolate was included in the analysis.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing and phenotyping for ESBL-EC were performed using the broth microdilution method based on the CLSI guidelines.[10] Eleven drugs were used for the antimicrobial susceptibility testing, including CTX, ceftazidime (CAZ), ceftriaxone (CRO), cefoperazone/sulbactam (CPS), piperacillin/tazobactam (TZP), cefepime (FEP), amikacin (AMK), ciprofloxacin (CIP), levofloxacin (LVX), imipenem (IPM) and meropenem (MEM). The interpretation breakpoint of CPS corresponded to that of cefoperazone in the study.

The control strains included *E. coli* ATCC 25922 and *Klebsiella pneumonia* (*K. pneumoniae*) ATCC 700603.

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Received 21 December 2014; revised 1 September 2015; accepted 4 September 2015; published online 30 September 2015

Molecular detection of ESBL

PCR was used to screen ESBL-EC isolates for $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}2}$ group, $bla_{CTX\text{-}M\text{-}8}$ group, $bla_{CTX\text{-}M\text{-}9}$ group and $bla_{CTX\text{-}M\text{-}25}$ group. Primer pairs of $bla_{CTX\text{-}M}$ will amplify the entire open reading frame (ORF) of the target gene. PCR was also used to screen for ESBL-EC isolates to detect bla_{TEM}, bla_{SHV} and bla_{OXA} (Table 1).

Purified PCR products were directly sequenced and were compared with the deposited sequences in NCBI (http://www.ncbi.nlm.nih.gov/). Finally, the subtypes of ESBL were confirmed by the Lahey system (www.lahey.org/studies/).

Conjugation assay

Resistance transfer of ceftazidime from some ESBL-EC strains was performed by using the broth culture conjugation method, as previously described.[11] Six ESBL-EC strains with high ceftazidime resistance (MIC $\geqslant 16\,mg\,l^{-1}$) were selected in the assay, including four strains with $bla_{CTX\text{-}M\text{-}15}$ and two strains with $bla_{CTX\text{-}M\text{-}55}$.

In brief, ESBL-EC strains carrying CTX-M type with ceftazidime resistance were selected as the donor, and *E.coli* strain J53 was used as the recipient that is resistant to sodium azide. After the 4 h mating time, transconjugants were selected by culturing on Mueller–Hinton agar plates containing ceftazidime ($2\,mg\,l^{-1}$) and sodium azide ($200\,mg\,l^{-1}$) at 37 °C for 16 h. The colony was selected by antimicrobial susceptibility testing to ceftazidime and detection of the CTX-M genes was performed as described above.

RESULTS

Distribution and antimicrobial susceptibility testing of ESBL-EC

A total of 32 762 blood culture samples were collected over 6 years. Pathogens were detected in 13.9% (4552/32 762) of all samples. Of those, 18.8% (856/4552) of the samples were *E. coli* positive. Among the *E. coli* strains, 294 ESBL-EC isolates were confirmed (34.3%, 294/856). An increased trend in the isolation rate of ESBL-EC was observed in 17.2% (23/134) of samples from 2008, 29.0% (38/131) from 2009, 33.1% (44/133) from 2010, 37.3% (53/142) from 2011, 42.3% (66/156) from 2012 and 43.5% (70/161) from 2013.

Table 1 Primer pairs used for *bla* genes detection of ESBL-EC in PCR assay

Target genes	*Primer*	*Sequence(5′-3′)*	*Amplicon size (bp)*
$bla_{CTX\text{-}M\text{-}1}$ Group	Forward	ATGGTTAAAAAATCACTGCGYCAG	876
	Reverse	TTACAAACCGTYGGTGACGATTTTAG	
$bla_{CTX\text{-}M\text{-}2}$ Group	Forward	ATGATGACTCAGAGCATTCGCC	876
	Reverse	TCAGAAACCGTGGGTTACGATTTTC	
$bla_{CTX\text{-}M\text{-}8}$ Group	Forward	TTTTACTTTTTGTGCTGACTGTGAA-TACTTC	939
	Reverse	TTAATAACCGTCGGTGACGATTTTCG	
$bla_{CTX\text{-}M\text{-}9}$ Group	Forward	ATGGTGACAAAGAGAGTGCAACGG	873
	Reverse	CAGCCCTTCGGCGATGATTC	
$bla_{CTX\text{-}M\text{-}25}$ Group	Forward	ATGATGAGAAAAAGCGTAAGGCGG	876
	Reverse	TTAATAACCGTCGGTGACAATTCTGG	
bla_{TEM}	Forward	CCGCATACACTATTCTCAGAATG	440
	Reverse	CTCACCGGCTCCAGATTTATC	
bla_{SHV}	Forward	TGTATTATCTCCCTGTTAGCCACC	767
	Reverse	GTATCCCGCAGATAAATCACCA	
bla_{OXA}	Forward	GGCACCAGATTCAACTTTCAAG	256
	Reverse	ATCTCCAGAGAAGTCTTGATTTCC	

Abbreviations: CTX, cefotaxime; ESBL, extended-spectrum β-lactamase.

All 294 ESBL-EC isolates were resistant to CTX and CRO, and most of the strains (93.5%) were resistant to FEP. All of the ESBL-EC isolates were highly susceptible to CPS (86.4%), TZP (95.2%), AMK (90.5%), IPM (98.6%) and MEM (99.3%), whereas high resistance rates were observed with CIP (66.0%) and LVX (69.0%) over the 6-year study period. Intriguingly, ESBL-EC isolates showed significantly increased resistance to ceftazidime from 2008 to 2013 (34.8 to 68.6%) (Table 2). No change in ceftazidime MICs was found in ceftazidime-resistant ESBL-EC during the 6-year study period.

Molecular detection of ESBL

Approximately 95% of ESBL-EC isolates harbored the $bla_{CTX\text{-}M}$ gene, and 16.7% of the strains simultaneously carried two types of $bla_{CTX\text{-}M}$. The major types of $bla_{CTX\text{-}M}$ were $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}55}$, which accounted for 50.3%, 24.8% and 19.0%, respectively, of all strains during the 6-year study period. Compared with the decreased trend of $bla_{CTX\text{-}M\text{-}14}$, there was a significant trend in $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}55}$ that were observed from 2008 to 2013. Upon sequencing, it was observed that all $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}55}$ harbor the Asp240Gly substitution. Noticeably, the total prevalence of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}55}$ in ESBL-EC were higher than that of $bla_{CTX\text{-}M\text{-}14}$ in 2012 and 2013 (Table 2). All CTX-M producing isolates of CTX-M-14 without other types were sensitive to cefotaxime (MIC range $1–4\,mg\,l^{-1}$). Most ceftazidime-resistant strains (73.1%) contained $bla_{CTX\text{-}M\text{-}15}$ or $bla_{CTX\text{-}M\text{-}55}$, and all strains containing $bla_{CTX\text{-}M\text{-}15}$ or $bla_{CTX\text{-}M\text{-}55}$ were resistant to ceftazidime with high MIC (range from 16 to $64\,mg\,l^{-1}$).

Of the 294 isolates tested, 23.5% (69/294) carried bla_{TEM}, 1.0% (3/294) carried bla_{SHV} and no isolates contained bla_{OXA}. Sequence analysis of the PCR products demonstrated that all bla_{TEM} were $bla_{TEM\text{-}1}$, bla_{SHV} *or* $bla_{SHV\text{-}12}$. No significant trends were observed in the distribution of bla_{TEM} and bla_{SHV} in ESBL-EC from 2008 to 2013 (Table 2).

Transfer of ceftazidime resistance

Conjugation assay was used to transfer plasmids of ceftazidime resistance to *E. coli* strain J53, which was successful after confirming with six ESBL-EC strains with $bla_{CTX\text{-}M\text{-}15}$ or $bla_{CTX\text{-}M\text{-}55}$. All transconjugants had high resistance to ceftazidime with MIC $\geqslant 16\,mg\,l^{-1}$, and the corresponding $bla_{CTX\text{-}M}$ genes were also detected in the transconjugants using PCR.

DISCUSSION

ESBL-producing *Enterobacteriaceae* is a significant global public health threat worldwide, especially ESBL-EC infection in BSI. The ESBL production of the pathogens is the main mechanism of resistance to cephalosporins.

Although, there is no consensus definition of ESBL, many ESBL types have been proposed to be TEM type, SHV type, CTX-M type, ox-acillinase (OXA) type, Guiana extended-spectrum (GES) type or Vietnamese extended-spectrum-lactamase (VEB) type.[12] Historically, TEM type and SHV type ESBL were the predominant types found worldwide. Nevertheless, the molecular epidemiology and clinical features of CTX-M-type in *E. coli,* mainly CTX-M-14, have been frequently studied in many countries in recent years, including some regions of China.[13] Based on the recommendation of CLSI (M100-S20 document, 2010), the susceptibility results of *E. coli* for ceftazidime should be assessed according to MIC without considering ESBL production. In this study, the antimicrobial agent resistance rate and CTX-M genotypic profile were analyzed among ESBL-EC isolated from BSI during a 6-year study period from the university-affiliated

Table 2 Antimicrobial agent resistance rate and ESBL genotypic distribution of ESBL-EC isolates from 2008 to 2013 (%(*n*))

	2008 (23)	*2009 (38)*	*2010 (44)*	*2011 (53)*	*2012 (66)*	*2013 (70)*	*Total (294)*
Antimicrobial agent							
CTX	100 (23)	100 (38)	100 (44)	100 (53)	100 (66)	100 (70)	100 (294)
CRO	100 (23)	100 (38)	100 (44)	100 (53)	100 (66)	100 (70)	100 (294)
CAZ	34.8 (8)	39.5 (15)	54.5 (24)	56.6 (30)	69.7 (46)	68.6 (48)	58.2 (171)
FEP	91.3 (21)	92.1 (35)	93.2 (41)	94.3 (50)	93.9 (62)	94.3 (66)	93.5 (275)
CPS	13.0 (3)	13.2 (5)	13.6 (6)	13.2 (7)	13.6 (9)	14.3 (10)	13.6 (40)
TZP	4.3 (1)	5.3 (2)	4.5 (2)	5.7 (3)	4.5 (3)	4.3 (3)	4.8 (14)
AMK	8.7 (2)	10.5 (4)	9.1 (4)	9.4 (5)	10.6 (7)	8.6 (6)	9.5 (28)
CIP	60.9 (14)	73.7 (28)	63.6 (28)	71.7 (38)	63.6 (42)	62.9 (44)	66.0 (194)
LVX	65.2 (15)	73.7 (28)	68.2 (30)	75.5 (40)	68.2 (45)	64.3 (45)	69.0 (203)
IPM	0 (0)	2.6 (1)	0 (0)	1.9 (1)	1.5 (1)	1.4 (1)	1.4 (4)
MEM	0 (0)	2.6 (1)	0 (0)	0 (0)	0 (0)	1.4 (1)	0.07 (2)
ESBL type							
$bla_{\text{CTX-M-14}}$	65.2 (15)	60.5 (23)	61.4 (27)	54.7 (29)	39.4 (26)	40.0 (28)	50.3 (148)
$bla_{\text{CTX-M-15}}$	8.7 (2)	10.5 (4)	13.6 (6)	30.2 (16)	30.3 (20)	35.7 (25)	24.8 (73)
$bla_{\text{CTX-M-55}}$	8.7 (2)	13.2 (5)	13.6 (6)	20.8 (11)	24.2 (16)	22.9 (16)	19.0 (56)
$bla_{\text{CTX-M-123}}$	0 (0)	0 (0)	2.3 (1)	0 (0)	0 (0)	1.4 (1)	0.7 (2)
$bla_{\text{TEM-1}}$	21.7 (5)	23.7 (9)	22.7 (10)	24.5 (13)	21.2 (14)	25.7 (18)	23.5 (69)
$bla_{\text{SHV-12}}$	0 (0)	2.7 (1)	0 (0)	1.9 (1)	0 (0)	1.4 (1)	1.0 (3)
bla_{OXA}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Abbreviations: AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CPS, cefoperazone/sulbactam; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; LVX, levofloxacin; MEM, meropenem; TZP, piperacillin/tazobactam.

hospital of China. To our knowledge, our work represents the first study on the changes of the antimicrobial resistance and CTX-M type epidemiology of ESBL-EC after the release of the revised M100-S20 document.

In this study, the isolation rate of ESBL-EC in BSI significantly increased from 17.2% (2008) to 43.5% (2013), and similar results were also reported in other regions of China.[14] Our results also indicated that ESBL-EC were highly susceptible to carbapenem, β-lactam/β-lactamase inhibitor combinations and amikacin during the 6-year study period. These antimicrobial agents should be further studied to assess their efficacy against ESBL-EC infections in the region. Nearly all ESBL-EC isolates were resistant to the third-generation cephalosporins (cefotaxime and ceftriaxone) and cefepime, and only $<40\%$ isolates were susceptible to fluoroquinolones. No change in prevalence was observed in the resistance rate of these antibiotics from 2008 to 2013. Cephalosporin and fluoroquinolone are not considered as the effective choices for the treatment of patients with ESBL-EC infection in this region due to the relatively high mortality that is associated with ineffective therapy for BSI.[15]

According to the previous CLSI recommendations, all ESBL-EC isolates would have been reported as ceftazidime resistant, but some CTX-M-producing *E. coli* should be reported as susceptible to ceftazidime under the new recommendations in 2010. In this study, resistance rates to ceftazidime were significantly increased in ESBL-EC isolates over the 6-year study period (from 34.8% in 2008 to 68.6% in 2013). Notably, the resistance rate of ceftazidime rapidly increased after 2011.

CTX-M are the most dominant type found, and comprises a variety of subtypes in China. CTX-M-14 remains the most abundant type, although the detection rate of CTX-M-15 has been continuously increasing in recent years.[16,17] In this study, 294 ESBL-EC strains were collected to detect for ESBL types. We observed that most isolates (94.9%) harbored $bla_{\text{CTX-M}}$, and 16.7% of the strains carried two types of $bla_{\text{CTX-M}}$. The most commonly observed types were $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-55}}$, whereas $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-55}}$, which have been found to be rapidly spread from 2011 to 2013. The total distribution of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-55}}$ in ESBL-EC were higher than $bla_{\text{CTX-M-14}}$ from 2012 to 2013.

Ceftazidime is an effective antimicrobial agent against *E. coli* and is a substrate that is hydrolyzed by some types of enzymatic CTX-M.[18] The recent emergence of these variants suggests that the CTX-M enzymes, such as $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-123}}$, are evolving towards acquiring improved activity against ceftazidime.[19,20] The most prevalent CTX-M types (CTX-M-15 and CTX-M-55) that harbor the Asp240Gly substitution in the region confer high ceftazidime-hydrolyzing activity. In this study, isolates with CTX-M-14 were found to be more sensitive to ceftazidime than those containing other CTX-M types (CTX-M-15 and CTX-M-55). This could increase ceftazidime resistance in ESBL-EC due to changes of CTX-M type in the region.

Some epidemic plasmids have had a major role in the dissemination of $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ among *E. coli* and *K. pneumoniae* isolates.[16,17] In this study, conjugation assays yielded positive results, which suggests plasmid localization of the CTX-M genes. This also suggests that the strains are capable of transferring the CTX-M to disseminate ceftazidime resistance in the region.

In conclusion, the new ceftazidime breakpoint in M100-S20 document has not decreased the percentage of ceftazidime resistance over the 6-year study period in the region. The resistance rate to ceftazidime has significantly increased in ESBL-EC isolates, and ceftazidime-resistant ESBL-EC have been observed to rapidly spread after 2011 in the region. Increased levels of resistance could be due to higher rates of CTX-M types that possess high ceftazidime-hydrolyzing activity, mainly from those of CTX-M-15 and CTX-M-55 with plasmid spreading.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the National Science and Technology Major Project of China (2013ZX10004217).

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