

Original Article

Plasmid-Mediated AmpC β -Lactamase and Underestimation of Extended-Spectrum β -Lactamase in Cefepime-Susceptible Elevated-Ceftazidime-MIC Enterobacteriaceae Isolates

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SUMMARY: Phenotypic detection of extended-spectrum β -lactamase (ESBL) is important for public health and infection control; however, plasmid-mediated AmpC β -lactamases (pAmpCs) can interfere with the ESBL phenotyping. We focused on Enterobacteriaceae strains that were susceptible to cefepime but had a mildly elevated minimum inhibitory concentration (MIC) of ceftazidime and studied the effect of pAmpC on the ESBL phenotyping in this population. Genotyping of ESBL and pAmpC was performed on 528 clinical isolates of *Escherichia coli*, *Klebsiella* spp., and *Proteus* spp. with a ceftazidime MIC of ≥ 2 $\mu\text{g}/\text{mL}$ and cefepime MIC ≤ 8 $\mu\text{g}/\text{mL}$; these isolates were collected at Nagasaki University Hospital from January 2005 to March 2011. In this sample, 145 isolates (27.5%) tested positive for pAmpC (pAmpC group). The concordance rates of phenotypic and genotypic detection of ESBLs were 69.2% in the pAmpC group and 88.8% in the non-pAmpC group ($P = 0.04$). pAmpC was more commonly detected in isolates with non-CTX-M genes (5/53, 9.4%) than in isolates with CTX-M genes (8/121, 6.6%). Our data suggest that the presence of pAmpC increases the false negative detection of ESBL. When ESBL phenotyping is used, the underestimation of the prevalence of ESBL producers should be taken into account.

INTRODUCTION

Plasmid-mediated β -lactamases, such as extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases (AmpCs), have been spreading among Enterobacteriaceae strains worldwide (1–3). The expansion of these β -lactamases has been increasing the prevalence of cephalosporin-resistant Enterobacteriaceae. Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) no longer recommend routine testing for ESBLs in clinical microbiology laboratories (4,5); however, ESBL testing is still important for infection control and public health.

The increasing prevalence of plasmid-mediated AmpC (pAmpC) makes the identification of drug-resistant strains more complicated, especially for isolates that produce both AmpC β -lactamases and ESBLs. Importantly, ESBL screen-positive AmpC-producing strains yield negative confirmatory tests for detection of ESBL because AmpC β -lactamase-carrying strains are resistant to clavulanic acid (3). Erroneous identification of ESBL producers is disadvantageous for both the patients with

these infections and infection control. Indeed, outbreaks of pAmpC-carrying strains in hospitals have been reported (6,7).

Ceftazidime and cefepime are cephalosporins widely used against Enterobacteriaceae infections. Ceftazidime is also used for in vitro testing to screen ESBL producers, and a ceftazidime MIC of ≥ 2 $\mu\text{g}/\text{mL}$ is an ESBL-screening indicator (8). Generally, ESBL producers are widely resistant to cephalosporins; however, it is also known that some ESBL producers show cefepime MICs in the nonresistance range (≤ 8 $\mu\text{g}/\text{mL}$) (9,10). On the other hand, AmpC β -lactamases effectively hydrolyze ceftazidime but not cefepime (3). Therefore, Enterobacteriaceae strains that are susceptible to cefepime but have a mildly elevated MIC of ceftazidime can carry ESBL, pAmpC, or both.

In the present study, using the database of the microbiology laboratory in our hospital, we collected the clinical isolates including *Escherichia coli*, *Klebsiella* spp., and *Proteus* spp. with a ceftazidime MIC of ≥ 2 $\mu\text{g}/\text{mL}$ and cefepime MIC ≤ 8 $\mu\text{g}/\text{mL}$ and obtained their ESBL-phenotyping results. We also analyzed ESBL and pAmpC genes among the isolates and compared the data with their ESBL phenotypes.

MATERIALS AND METHODS

Clinical isolates: *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Proteus* spp. clinical isolates were selected from the microbiology laboratory database at Nagasaki University Hospital. A total of 8,299 isolates collected between January 2005 and March 2011 were

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included in this study. The numbers of *E. coli*, *Klebsiella* spp., and *Proteus* spp. isolates were 5,239 (63.1%), 2,653 (32.0%), and 407 (4.9%), respectively.

Inclusion criteria: We used ceftazidime as a marker of reduced cephalosporin susceptibility because only MICs of ceftazidime among ESBL-screening parameters were available for all isolates throughout the study period. The isolates with a ceftazidime MIC of ≥ 2 $\mu\text{g}/\text{mL}$ and cefepime MIC ≤ 8 $\mu\text{g}/\text{mL}$ were included in the study (cefepime-susceptible, elevated-ceftazidime-MIC isolates). According to CLSI M100-S23 breakpoint criteria, the isolates were considered cefepime-susceptible at ≤ 8 $\mu\text{g}/\text{mL}$. Antimicrobial susceptibility testing was performed on a VITEK II (BD, Franklin Lakes, NJ, USA) or Phoenix (BD, USA) according to CLSI guidelines. Phenotypically confirmed ESBL producers (ph-ESBLs) were identified by a confirmatory test detecting ESBLs using the β -lactamase inhibitor clavulanic acid.

Extraction of plasmid DNA: The isolates were inoculated onto a blood agar plate and incubated at 36°C for 24 h. Then, a few colonies were resuspended in 200 μL of distilled water, and the suspension was heated at 98°C for 10 min. After centrifugation at $14,000 \times g$ for 10 min, the supernatant was employed as the DNA template for multiplex polymerase chain reaction (PCR) and for sequencing. The extract could contain both genomic DNA and plasmid DNA.

Detection of ESBL genes and pAmpCs: ESBL genes, such as TEM, SHV, CTX-M-1 group, CTX-M-2 group, and CTX-M-9 group were detected by PCR as previously described (2). The isolates carrying these genes, except for *E. coli* isolates positive for TEM only and *K. pneumoniae* isolates positive for SHV only, were considered genotypically confirmed ESBL producers (g-ESBLs).

For *E. coli* isolates positive for TEM only and *K. pneumoniae* isolates positive for SHV only, the sequence of these β -lactamases was determined by Sanger sequencing by means of the following primer sets: for TEM, sense primer, 5'-TCCGCTCATGAGACAATAA CC-3'; anti-sense primer, 5'-TTGGTCTGACAGTTAC-CAATGC-3'; for SHV, sense primer, 5'-TGCGTTATAT TCGCCTGTGT-3'; anti-sense primer, 5'-GGGGTATC

CCGCAGATAAAT-3'. After the sequence was evaluated according to the functional classification (11), the isolates with group 2be enzymes were included into the group of g-ESBLs.

For the pAmpC screening, we used a multiplex PCR method to detect *mox*, *cit*, *lat*, *dha*, *act*, and *fox*, as previously reported (12). Positive controls were kindly provided by N. D. Hanson. The PCR products were analyzed by 2% agarose gel electrophoresis.

Statistical analysis: We compared the numbers and percentages of isolates categorized as ph-ESBLs and g-ESBLs in the pAmpC and non-pAmpC groups and analyzed the distribution of pAmpC isolates according to the cefepime MIC. The chi-square test was conducted to analyze the significance of differences in the percentage between groups. *P* values less than 0.05 were considered statistically significant.

Ethical standard: This study does not contain any specimens and information which need ethical approvals.

RESULTS

Cefepime-susceptible elevated-ceftazidime-MIC strains: During the study period, 528 (6.4%) of the 8,299 isolates showed ceftazidime MICs of ≥ 2 $\mu\text{g}/\text{mL}$ and cefepime MICs of ≤ 8 $\mu\text{g}/\text{mL}$ (Fig. 1), and the number (percentage) of *E. coli*, *Klebsiella* spp., and *Proteus* spp. isolates were 417 (8.0%), 97 (3.7%), and 14 (3.4%), respectively. Among the 528 isolates, there were 230 (43.6%) ph-ESBLs and 174 (33.0%) g-ESBLs. The numbers of ph-ESBLs among the *E. coli*, *Klebsiella* spp., and *Proteus* spp. cefepime-susceptible, elevated-ceftazidime-MIC isolates were 175 (42.0%), 55 (56.7%), and 0 (0.0%), respectively. The numbers of g-ESBLs among the *E. coli*, *Klebsiella* spp., and *Proteus* spp. with ceftazidime MICs of ≥ 2 $\mu\text{g}/\text{mL}$ and cefepime MICs of ≤ 8 $\mu\text{g}/\text{mL}$ were 133 (31.9%), 38 (39.2%), and 3 (21.4%), respectively (Table 1). Thus, the isolates were categorized into the following 4 groups: ph-ESBL/g-ESBL ($n = 152$), non-ph-ESBL/g-ESBL ($n = 22$), ph-ESBL/non-g-ESBL ($n = 78$), and non-ph-ESBL/non-g-ESBL ($n = 276$; Fig. 1).

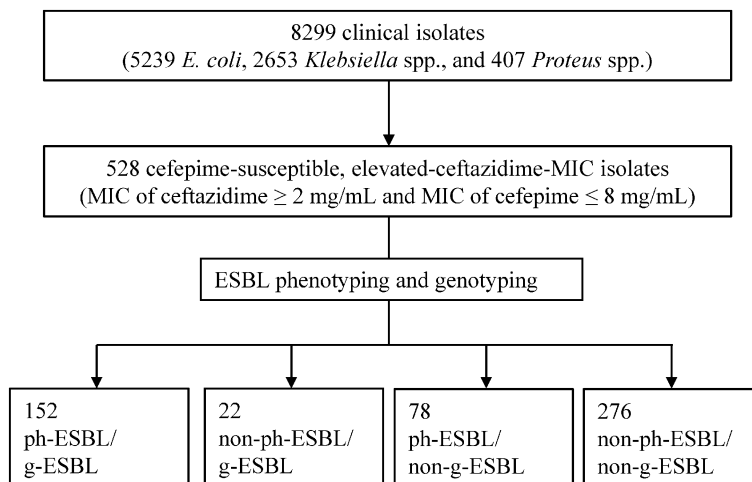


Fig 1. The procedures in this study.

Table 1. ESBL genotypes in the cefepime-susceptible elevated-ceftazidime-MIC isolates

ESBL genotypes	<i>E. coli</i> n (%)	<i>Klebsiella</i> spp. n (%)	<i>Proteus</i> spp. n (%)	All strains n (%)
TEM, 2be ¹⁾	—	4 (5.3)	3 (100.0)	7 (2.3)
TEM, others	93 (41.2)	NA	NA	93 (30.6)
SHV, 2be ²⁾	13 (5.8)	12 (16.0)	—	25 (8.2)
SHV, others	NA	37 (49.3)	NA	37 (12.2)
CTX-M-1 group	1 (0.4)	—	—	1 (0.3)
CTX-M-2 group	1 (0.4)	—	—	1 (0.3)
CTX-M-9 group	53 (23.5)	2 (2.7)	—	55 (18.1)
TEM, SHV	6 (2.7)	15 (20.0)	—	21 (6.9)
TEM, CTX-M-1 group	2 (0.9)	—	—	2 (0.7)
TEM, CTX-M-2 group	6 (2.7)	—	—	6 (2.0)
TEM, CTX-M-9 group	50 (22.1)	—	—	50 (16.4)
SHV, CTX-M-2 group	—	2 (2.7)	—	2 (0.7)
SHV, CTX-M-9 group	1 (0.4)	1 (1.3)	—	2 (0.7)
TEM, SHV, CTX-M-9 group	—	2 (2.7)	—	2 (0.7)
Total	226 (100.0)	75 (100.0)	3 (100.0)	304 (100.0)

¹⁾: sequenced for *E. coli* positive for TEM only.

²⁾: sequenced for *K. pneumoniae* positive for SHV only.
NA, not applicable.

Table 2. ESBL-carriers in the pAmpC and non-pAmpC groups

ESBL phenotype/genotype	pAmpC group n (%)	non-pAmpC group n (%)
ph-ESBL/g-ESBL	9 (6.2)	143 (37.3)
non-ph-ESBL/g-ESBL	4 (2.8)	18 (4.7)
ph-ESBL/non-g-ESBL	10 (6.9)	68 (17.8)
non-ph-ESBL/non-g-ESBL	122 (84.1)	154 (40.2)
Total	145 (100.0)	383 (100.0)

Genotyping of ESBL genes: A total of 391 ESBL genes were detected in 304 strains, and the number (percentage of all ESBL genes) of TEM, SHV, CTX-M-1 group, CTX-M-2 group, and CTX-M-9 group was 181 (46.3%), 89 (22.7%), 3 (0.8%), 9 (2.3%), and 109 (27.9%), respectively (Table 1). Among the *E. coli* isolates, group TEM alone was the most prevalent (41.2%) followed by group CTX-M-9 group alone (23.5%), and group TEM plus CTX-M-9 group (22.1%). Among the *Klebsiella* spp. isolates, SHV alone (65.3%) and TEM plus SHV (20.0%) were observed most frequently. All the *Proteus* spp. isolates carried TEM alone. The number of group 2be TEM genes in *E. coli* and group 2be SHV genes in *K. pneumoniae* was 0 and 12, respectively.

Detection and genotyping of pAmpCs: To confirm the relation between ESBLs and pAmpCs, we screened the strains for pAmpCs. Of the 528 isolates that were classified as cefepime-susceptible elevated-ceftazidime-MIC, 145 (27.5%) carried pAmpC. pAmpCs were detected in 30.9% of the *E. coli* isolates, 4.1% of the *Klebsiella* spp. isolates, and 85.7% of the *Proteus* spp. isolates. The numbers of strains carrying *mox*, *cit*, *lat*, *dha*, *act*, or *fox* genes were 0 (0.0%), 139 (95.9%), 0 (0.0%), 6 (4.1%), 0 (0.0%), and 0 (0.0%), respectively (Table 2).

ESBL-carrying strains in the pAmpC and non-pAmpC groups: The prevalence of ESBLs among iso-

lates with and without pAmpC (the pAmpC and non-pAmpC groups, respectively) was compared (Table 2). The percentages of g-ESBL isolates in the pAmpC and non-pAmpC groups were 9.0% (13/145) and 42.0% (161/383), respectively. In contrast, the percentage of ph-ESBL isolates in the pAmpC group was 13.1% (19/145), whereas this percentage in the non-pAmpC group was 55.1% (211/383). The percentage of ph-ESBLs concordant with g-ESBL (ph-ESBL/g-ESBL) in the pAmpC group was 6.2%, whereas that in the non-pAmpC group was 37.3%. Thus, the percentage of accurately identified ph-ESBLs was significantly lower in the pAmpC group ($P = 0.01$, 69.2% in the pAmpC group vs. 88.8% in the non-pAmpC group, Table 3). Additionally, the percentage of isolates that were misidentified as non-ph-ESBLs (non-ph-ESBL/g-ESBL) in the pAmpC group was significantly lower than that in the pAmpC group vs. 4.7% in the non-pAmpC group; $P < 0.05$).

ESBL genotypes in the pAmpC and non-pAmpC groups: To analyze the relation between pAmpCs and ESBL genotypes, the ESBL genotypes in the pAmpC and non-pAmpC groups were compared (Table 3). In the pAmpC group ($n = 13$), 6 isolates (46.2%) had one ESBL gene and 7 (53.8%) had 2 or more ESBL genes. In the non-pAmpC group ($n = 161$), 83 isolates (51.6%) carried one ESBL gene, and 78 (48.4%) carried 2 ESBL genes. Of the 109 CTX-M-9 group-carrying isolates, 106 (97.2%) were in the non-pAmpC group. pAmpC was more frequently detected among isolates without CTX-M (5/53, 9.4%) than among isolates with CTX-M (8/121, 6.6%).

Among the isolates with one ESBL gene, the percentage of ph-ESBLs strains was significantly lower in the pAmpC group than in the non-pAmpC group (33.3% in the pAmpC group vs. 72.3% in the non-pAmpC group; $P = 0.045$). In contrast, there were no significant differences in the percentages of ph-ESBLs strains among isolates with at least 2 ESBL genes between the 2 groups

Table 3. ESBL genotypes in the pAmpC and non-pAmpC groups

ESBL genes	Number of ESBL, phenotype/ genotype (%)		P value
	pAmpC	non-pAmpC	
Single			
TEM	0/3 (0.0)	0/4 (0.0)	NS
SHV	1/2 (50.0)	22/23 (95.6)	0.02
CTX-M-1 group	—	1/1 (100.0)	—
CTX-M-2 group	—	0/1 (0.0)	—
CTX-M-9 group	1/1 (100.0)	47/54 (87.0)	NS
Single total	2/6 (33.3)	60/83 (72.3)	0.045
Multiple			
TEM, SHV	—	16/21 (76.2)	—
TEM, CTX-M-1 group	1/1 (100.0)	1/1 (100.0)	NS
TEM, CTX-M-2 group	4/4 (100.0)	2/2 (100.0)	NS
TEM, CTX-M-9 group	—	50/50 (100.0)	—
SHV, CTX-M-2 group	—	2/2 (100.0)	—
SHV, CTX-M-9 group	—	2/2 (100.0)	—
TEM, SHV, CTX-M-9 group	2/2 (100.0)	—	—
Multiple total	7/7 (100.0)	73/78 (93.9)	NS
Total	9/13 (69.2)	143/161 (88.8)	0.04

NS, not significant.

(100.0% in the pAmpC group vs. 93.9% in the non-pAmpC group). Among the ESBL genotypes, the proportion of isolates having SHV alone that were correctly identified as ph-ESBL in the pAmpC group was significantly lower than that in the non-pAmpC group (50.0% in the pAmpC group vs. 95.6% in the non-pAmpC group; $P = 0.02$). The percentages of correctly identified isolates with and without CTX-M were 93.2% (109/117) and 73.6% (39/53), respectively ($P < 0.01$).

DISCUSSION

Generally, it has been estimated that pAmpCs are less prevalent than ESBLs in Enterobacteriaceae. In a surveillance study of clinical isolates in Japan, the proportions of AmpC-producing isolates were 0.12% for *E. coli*, 0.13% for *K. pneumoniae*, 0.17% for *K. oxytoca*, and 0.08% for *Proteus mirabilis* (13). In the United States, among isolates with a MIC ≥ 2 $\mu\text{g/mL}$ for cephalosporins, including ceftazidime, pAmpC genes have been found in 4.0% of *E. coli* isolates, 8.5% of *K. pneumoniae* isolates, and 6.9% of *K. oxytoca* isolates (14). In the present study, we observed higher prevalence of pAmpC-carrying isolates (27.5%) as compared to these previous reports, implying that our criteria for isolate inclusion (a ceftazidime MIC of ≥ 2 $\mu\text{g/mL}$ and cefepime MIC of ≤ 8 $\mu\text{g/mL}$) effectively included pAmpC-carrying strains.

The effect of cefepime on the treatment of cefepime-susceptible ESBL bacteremia has been addressed in some studies (15,16). Although the contribution of pAmpC was not assessed in these reports, cefepime was inferior to carbapenem for those infections. The drug susceptibility pattern of pAmpC is partly similar to that of ESBL (3), and isolates cohabiting these β -lactamases require careful consideration when one deciphers the results of drug susceptibility tests (17,18). In the present

study, isolates that carried ESBL genes but were phenotypically identified as ESBL nonproducers were significantly more frequently observed in the pAmpC group than in the non-pAmpC group. This finding suggests that pAmpC can mask the ESBL phenotype.

Few studies are available on the combination of ESBLs and pAmpCs. However, pAmpC appears to be detected concurrently with TEM or SHV (3), and it has been reported that 50% of pAmpC-positive isolates are also TEM-positive (17). The present study did not reveal a possible relation between pAmpC and TEM; however, most isolates possessing CTX-M did not have pAmpC. Therefore, pAmpC may be incompatible with CTX-M in cefepime-susceptible strains with an elevated ceftazidime MIC.

The poor compatibility between CTX-M and pAmpCs appears to conversely correlate with correct phenotypic identification of ESBL. However, the 8 CTX-M isolates cohabiting pAmpC were all identified as ph-ESBLs, suggesting that the ESBL confirmatory test can effectively detect the CTX-M ESBL. These different findings can be explained, in part, by the in vitro cephalosporin-hydrolytic activity of these lactamases, which is the strongest for CTX-M, followed by TEM and SHV (3). Thus, the masking effect of pAmpC in ESBL testing can vary according to the ESBL genotype. Nonetheless, further investigation is necessary to fully determine the relations between false negatives and individual ESBL and pAmpC genotypes because this study was biased in that our cefepime susceptibility criterion may have enriched the study sample with isolates carrying TEM alone (19).

There are some limitations of this study. First, our findings may be limited to the isolates with a ceftazidime MIC of ≥ 2 $\mu\text{g/mL}$ because we did not use MICs of other cephalosporins such as cefpodoxime and cefotaxime as inclusion criteria. Second, we did not investigate all isolates collected during the study period. Thus, the overall proportion of isolates having pAmpCs is unknown. Third, we did not perform in vitro interaction studies on the hydrolytic activities of the pAmpCs and ESBLs. Therefore, further research is needed to determine whether pAmpCs actually inhibit ESBLs in confirmatory tests of each cohabiting isolate. Lastly, because this was a single-center study, the results should be confirmed using isolates from other hospitals to determine whether our criteria—ceftazidime MIC ≥ 2 $\mu\text{g/mL}$ and cefepime MIC ≤ 8 $\mu\text{g/mL}$ —are appropriate for effective detection of pAmpCs.

In conclusion, Enterobacteriaceae isolates with a ceftazidime MIC of ≥ 2 $\mu\text{g/mL}$ and a cefepime MIC of ≤ 8 $\mu\text{g/mL}$ showed high prevalence of pAmpC. The fact that ESBL production may be masked by pAmpC in strains with a ceftazidime MIC of ≥ 2 $\mu\text{g/mL}$ and a cefepime MIC of ≤ 8 $\mu\text{g/mL}$ should be taken into account. These findings are important for the patients as well as infection control and public health considerations when phenotypic ESBL detection is performed.

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Conflict of interest None to declare.

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