Occurrence of Plasmid-mediated AmpC β-lactamases among *Enterobacter cloacae, Serratia marcescens* and *Citrobacter freundii* in China

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ABSTRACT

Background: A total of 775 consecutive non-duplicate isolates of *Enterobacteriaceae* (534 *E. cloacae*, 137 *S. marcescens* and 104 *C. freundii*) were collected from 2005 to 2014 at 34 hospitals in Anhui Province, China.

Material and Methods: The detection of plasmid-mediated AmpC β-lactamases (pAmpCs) was based on the inhibitor screening method. Then, the association with other β-lactamases and mobile genetic elements, like class 1 integron, IS*CR1* and IS*Ecp1*, were analyzed by molecular biology method.

Results: Among the total of 775 isolates, the prevalence of pAmpC was discovered to be 8.1% (63/775) overall. In this study, the presence of pAmpCs was significantly linked to other β-lactamases and mobile genetic elements, among which $bla_{\text{CTX-M}}$ and class 1 integron were the most prevalent genotype.

Conclusion: Therefore, various combinations of β-lactamases and mobile genetic elements were detected in the study. Plasmids were demonstrated to be the main vehicles for the dissemination of pAmpC genes.

Keywords: class 1 integron, *Enterobacteriaceae,* plasmid-mediated AmpC β-lactamases

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INTRODUCTION

The β-lactams are a critically important class of antimicrobial agents utilized worldwide for the treatment of serious hospital- and community-acquired infections (1). The predominant mechanism for resistance to these antibiotics in gram-negative bacteria is the production of various plasmid-mediated β-lactamases, especially Ambler class A and class C β-lactamase genes. Plasmid-mediated AmpC β-lactamases (pAmpCs) are derived from chromosomal *ampC* genes of several members of the family *Enterobacteriaceae*, including *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii*, and *Hafnia alvei*. These pAmpCs have been reported from various clinical isolates with increasing frequency (2). *E. cloacae*, *S. marcescens* and *C. freundii* are important nosocomial pathogens which naturally harbour chromosomal *ampC* genes. The data regarding coproduction of extended- spectrum β-lactamases (ESBLs) and pAmpCs in *E. cloacae*, *S. marcescens* and *C. freundii* are fragmentary (3-5). Several studies have described that the dissemination of genes encoding plasmid-mediated β-lactamase is mediated by mobile genetic elements carried by large transferable plasmids (6, 7). In light of the above facts, the present extensive study was planned to determine the prevalence of pAmpCs, the co-presence of ESBLs and mobile genetic elements, like class 1 integrons, IS*CR1* and IS*Ecp1* in *E. cloacae*, *S. marcescens* and *C. freundii* isolates.

MATERIALS AND METHODS

Bacterial isolates

A total of 775 consecutive non-duplicate isolates of *Enterobacteriaceae* (534 *E. cloacae*, 137 *S. marcescens* and 104 *C. freundii*) were collected from 2005 to 2014 at 34 hospitals in Anhui Province, China. Anhui Province locates in eastern China with the permanent population of

about 60.829 million and covers the area of 139,600 square kilometers. All hospitals were more than 800 beds. Species identification was performed with the Vitek 2 system (bioMérieux, Marcy l'Étoile, France) and confirmed with API 20E identification (bioMérieux, Marcy l'Étoile, France).

Antimicrobial susceptibility testing

For the clinical isolates and transconjugants analyzed in this study, the minimum inhibitory concentrations (MICs) for cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), imipenem (IPM), piperacillin-tazobactam (TZP), ciprofloxacin (CIP) and amikacin (AMK) were determined by an agar dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline, 2014 (8). The antimicrobial agents were provided as powders by National Institute for the Control of the Pharmaceutical and Biological Products, China. Quality control strains were *E. coli* ATCC 25922 and *E. coli* ATCC 35218 every batch of clinical isolates to ensure accurate and comparable performance of assays. Detection of ESBL confirmatory tests completed on the transconjugants was carried out with the Cation-adjusted Mueller-Hinton Broth Dilution Method as described by CLSI recommended.

Conjugation experiments

Conjugation was done as described by Barguigua using sodium azide-resistant *E. coli* J53 (*E. coli* J53AZ ^{R}) as the recipient strain (9). Donor strains in the logarithmic phase of growth were mixed with recipients in early stationary phase at a 1:10 ratio in Muller-Hinton broth (Oxiod, UK), and the conjugation mixtures were incubated at 37 ℃ for 14 h. Transconjugants were selected on tryptone soya agar supplemented with sodium azide (100μg/ml, to inhibit growth of the donor strain) and FOX (16μg/ml, to inhibit growth of the recipient strain), which were incubated for approximately 20 h at 37℃. Tranconjugants were identified with

API 20E and Vitek-2 system. Transconjugants producing AmpC-type β-lactamases were detected by PCR using purified plasmid DNA as the template, ultimately sequenced and tested for susceptibility as described above for the clinical isolates.

Investigation of resistance determinants

The detection of AmpC β-lactamase was based on the AmpC enzyme inhibitor screening method (10). Plasmids DNAs were extracted from the AmpC screened- positive isolates and transconjugants using the Qiagen plasmid purification kit (Qiagen, Hilden, Germany). Genes encoding 6 phylogenetic groups of acquired AmpC enzymes were sought with a multiplex PCR assay (11). Different β-lactamase genes (including $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} and bla_{OXA}) and mobile elements such as class 1 integrons, IS*CR1* and IS*Ecp1* were analyzed by PCR using primers as described previously for the isolates carrying pAmpCs (9, 12, 13). Detection of the bla_{IPM} in pAmpCs producers was performed by PCR amplification. The purified PCR products were ligated with pUC118-Teasy vectors (TaKaRa, China), which were amplified in *E. coli* DH5a. All nucleotide sequences were sequenced by the dideoxy chain termination method using an ABI Prism 3730 sequence analyzer (Applied Biosystems, Foster City, CA). Sequence alignment was compared with the GenBank nucleotide database using the nucleotide BLAST program. The sequences of class 1 integron were analyzed by PCR-RFLP. After culturing the pAmpCs producers and ultrasonication, outer membrane proteins were extracted. Then, SDS-PAGE electrophoresis was performed and stained with Coomassie brilliant blue. The difference between the outer membrane proteins of clinical isolates was compared with *E. cloacae* ATCC 13047.

Southern hybridizations of transconjugants

The plasmids extracted from pAmpCs producers and their transconjugants by the rapid alkaline lysis protocol were digested with *Pst* I restriction enzyme (TaKaRa, China). The

plasmid size was determined by comparison with plasmids of *E. coli* V517 (54, 5.6, 5.1, 3.9, 3.0, 2.7, and 2.1 kb). DNA restriction fragment length polymorphisms were analyzed by the electrophoresis on the 0.8% agarose gel at 45 V for 16 h at 20℃, transferred onto a nylon membrane (Amersham Biosciences, UK) with positive ion (Hybond-N^+) overnight by the Southern hybridization technique. The membrance fixed by UV exposure was hybridized with PCR- generated probes whose sequences were specific internal regions of the $bla_{\text{DHA-1}}$, $bla_{\text{ACT-1}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{OXA-1}}$ genes fragments, respectively. The nonradioactive enhanced chemilumine-scence random prime kit (Boster, China) was used to label the DNA probes (20 ng/mL) and for detection.

RESULTS

Rates of resistance to CTX, CAZ, FOX, FEP, IPM, TZP, CIP and AMK in relation to total clinical isolates were 62.3%, 47.0%, 63.7%, 5.6%, 13.4%, 13.3%, 22.4% and 48.2%, respectively. Among the total of 775 clinical isolates, the prevalence of pAmpC carrier was found to be 8.1% (63/775) overall through the phenomenon of distorted inhibition zone around cefoxitin disk by the modified Hodge test. These pAmpCs were found in 54 of 534 *E. cloacae*, 5 of 137 *S. marcescens* and 4 of 104 *C. freundii*. Conjugative transfer of *ampC* gene to *E. coli* J53AZ ^{R} was positive for 53 of the 63 selected isolates (84.1%), including 46 *E. cloacae*, 5 *S. marcescens* and 2 *C. freundii*, which suggested that the dissemination of AmpC determinants was mostly due to the transmission of plasmids. All pAmpCs producers tested were IMP sensitive except for 2 *E. cloacae* which produced IMP-1 metalloenzyme and lost outer membrane porin protein. MICs of FOX towards 63 pAmpCs producers ranged from 64 mg/L to >256 mg/L. In addition, several of pAmpCs producers showed resistance to CIP and AMK. 53 transconjugants exhibited an increase in the MICs of β-lactams compared to the recipients. The transconjugants as well as pAmpCs producers present the similar resistance spectrum except for fluoroquinolones (Table 1). With regard to the genotypes, the most

prevalent pAmpCs producers were 39 *bla*$_{\text{DHA-1}}$, followed by 24 *bla*$_{\text{ACT-1}}$. Then, the *bla*$_{\text{ampC}}$ genotypes of transconjugants were *bla*$_{\text{DHA-1}}$ (n=37), next to *bla*$_{\text{ACT-1}}$ (n=23) (Table 2).

Different other β-lactamases genes of 63 pAmpCs producers and 53 transconjugants screened by PCR using specific primers and identified by DNA sequencing were summarized in Table 3. Out of the pAmpC-positive isolates and transconjugants, a total of 23 *E. cloacae* and 19 transconjugants from *E. cloacae* were found to carry at least one of these β-lactamase genes. The dominant β-lactamase genes in this study were TEM-1 and SHV-5 genes, followed by CTX-M-14 and CTX-M-15. Notably, 12 isolates and 7 transconjugants harboring pAmpCs carried more than one type of other β-lactamases in different combinations. ESBL phenotypic confirmatory testing for 19 transconjugants from *E. cloacae* showed the prevalence were only 10.53%. However, there were no positive results of ESBL found in other 34 transconjugants.

In 16 transconjugants carrying several of other β-lactamases genes, a single plasmid about 120-150kb was extracted from per transconjugant. For a given restricted digestion, the subsequent Southern hybridization was with *bla*$_{\text{DHA-1}}$, *bla*$_{\text{ACT-1}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{SHV-5}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$-specific probes. The restricted DNA fragments of 16 transconjugants were encoded by the same molecular weight fragment, approximately 70-90 kb. Corresponding to 16 pAmpCs producers, there was a unique plasmid of about 120-150kb detected from every clinical strain. Plasmid profiles were ran and then clinical isolates on the same blots as the transconjugants were used by Southern blot. The other 3 transconjugants carried 2 plasmids, which were the sizes of 120-140kb and 60-80kb, respectively. Corresponding to 3 clinical strains, 2 plasmids about 120-140kb and 60-80kb were found from per pAmpCs producers. For 3 transconjugants carrying *bla*$_{\text{OXA-1}}$, plasmids of approximately 60-80 kb were hybridized with the *bla*$_{\text{OXA}}$-specific probes. The electrophoretic display showed indistinguishable restriction patterns. Compared with other β-lactamases, it concluded that OXA-1 β-lactamase

was mediated by the different plasmid. The same molecular weight fragment as the clinical strains, approximately 60-80 kb, was detected in their transconjugants. To the transconjugant carrying $bla_{\text{DHA-1}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{OXA-1}}$, $bla_{\text{DHA-1}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-14}}$ were encoded in the same molecular weight fragment, approximately 50 kb, with specific probes. The same gels as the transconjugants with the clinical strains were detected with Southerns. Thus, $bla_{\text{DHA-1}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-14}}$ were also encoded in the identical plasmid in the isolates. To another transconjugant with $bla_{\text{DHA-1}}$, $bla_{\text{SHV-5}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{OXA-1}}$, $bla_{\text{DHA-1}}$, $bla_{\text{SHV-5}}$ and $bla_{\text{CTX-M-14}}$ were also encoded in the same molecular weight fragment, approximately 55 kb, with specific probes. In the clinical strains, the same blot about 55kb as the transconjugants was observed in the hybridization image of restriction fragment.

By performing PCR, a total of pAmpCs positive isolates harboring *intl1* integrase gene, IS*CR1* and IS*Ecp1* were detected in 47.6% (30/63), 39.7% (25/63) and 19.0% (12/63) respectively. Various combinations of observed genetic elements were more often detected in our study and *intI1* integrase gene plus IS*CR1* was found to be the most common combination in 11 pAmpC producers, followed by *intI1* plus IS*CR1* plus IS*Ecp1* in 10 pAmpC-positive clinical strains. None of the isolates harboured only IS*Ecp1* or IS*CR1* plus IS*Ecp1* combination. Highly similar frequencies of occurrence (55.6%) to *intl1* integrase gene were observed in 53 transconjugants, followed by IS*CR1* (47.2%) and IS*Ecp1* (22.6%). The structure of *intI1-*bla_{ampC}- IS*CR1-bla* in integrons is the most frequently detected in 11 transconjugants carrying 2 kinds of β-lactamases genes. Then, the other 8 transconjugants encoding several of β-lactamases genes presented the structural forms of *intI1-*bla_{ampC}*-bla*-IS*CR1*-IS*Ecp1 -bla.*

DISCUSSION

To the best of our knowledge, this is the first systematic molecular survey reporting the prevalence of pAmpCs *E. cloacae, S. marcescens* and *C. freundii* isolates. A prevalence rate of pAmpCs was 8.1% overall for these chromosomal AmpC- producing isolates which implied the extensive dissemination of pAmpC-producing bacteria in China. Regrettably, many studies lay emphasis on inducible AmpC- producers or derepressed AmpC mutants and neglect pAmpCs that are often expressed in large amounts and are encoded by transposons that can be easily transferred among bacteria. This study demonstrated that most of the pAmpCs genes were successfully transferred on transferable plasmids to the recipients. An increase in the MICs of β-lactam was detected in the transconjugants compared to the recipients. It is suggested that the dissemination of the pAmpCs is mostly due to the transmission of plasmids by horizontal exchange. Consequently, the rapid spread of pAmpCs by plasmids raises clinical concern among *Enterobacteriaceaes*, especially *K. pneumoniae* and *E. coli* which are known for lacking or poorly expressing chromosomal AmpC β-lactamase genes (2). We detected a dominance of DHA-1 which had been mostly reported from Asia. It had been found existing in the plasmids of these chromosomal AmpC-producing *Enterobacteriaceaes* as well (3, 14). bla_{MIR} and bla_{ACT} previously were thought to be 85 to 87% identity with most chromosomal AmpC enzymes in *E. cloacae* (15). However, conjugative plasmid-mediated bla_{MIR} and bla_{ACT} were detected in *E. cloacae* in our study, which were rarely reported before.

Several researches have shown a close association between pAmpCs and ESBLs (3). We observed that a total of 23 isolates can produce both pAmpCs and β-lactamases simultaneously, which is in accordance with previous reports. The coexistence of different classes of β-lactamases in a single isolate can cause failure of treatment because these combinations restrict the range of available therapeutic options to some extent (16). IS*CR1* elements have been found adjacent to a number of cephalosporinase genes, including class A

β-lactamase genes especially CTX-M and class C β-lactamase such as DHA, CMY and MOX (17). IS*CR1* appears to be unusual in the sense that it is almost always found in a complex class 1 integron structure, which has been the most prevalent combination in our study. We found that all IS*Ecp1*-positive isolates produce both *intI1* integrase gene encoded in a class 1 integron and IS*CR1*. In a recent study by Bae Ik *et al*, it was shown that a $bla_{\text{CTX-M-14}}$ gene was found on a complex class 1 integron and proceeded by a partial copy of IS*Ecp1* in addition to a complete copy of IS*CR1* element in *E. coli* contained 3 β-lactamase genes including $bla_{\text{DHA-1}}$, $bla_{\text{SHV-12}}$, and $bla_{\text{CTX-M-14}}$ (18). It can be that this new complex class integron composed of parts of a new IS*CR1*/IS*Ecp1*-associated fragment may have occurred among these pAmpC-producing isolates in China. The various antibiotic resistance genes existing in the same isolate can be carried by different mobile elements. Moreover, several studies have demonstrated that the IS*Ecp1* or IS*CR* element appeared to act as a strong activator for $bla_{\text{CTX-M}}$ gene expression (19). Since 16 transconjugants containing CTX-M type beta-lactamases, it would be possible that plasmids carrying $bla_{\text{CTX-M}}$ are responsible for transferability. *bla*ampC from chromosome jumped into plasmids carrying $bla_{\text{CTX-M}}$, which lead to the result of plasmid-mediated transferring *bla*ampC.

In conclusion, this is the first report describing the prevalence of pAmpCs among chromosomal AmpC-producing isolates. The concurrent occurrence of β-lactamases and the frequent association with mobile genetic elements, indicate a complex antibiotic resistance mechanism in these *Enterobacteriaceaes*. In our study, although plasmids were demonstrated to be the main vehicles for the dissemination of pAmpC genes among different bacteria, various mobile genetic elements may also play an important role in the prevalence of these enzymes.

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AUTHORS' NOTE

There are competing interests and no need to ethical approval.

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Table 1. MICs range for pAmpC producers and their transconjugants

Organism (the number of bacteria)	MIC range (CTX)	MIC_{50} (CTX)	MIC range (CAZ)	MIC_{50} (CAZ)	MIC range (FOX)	MIC_{50} (FOX)	MIC range (FEP)	MIC_{50} (FEP)	MIC range (IPM)	MIC_{50} (IPM)	MIC range (TZP)	MIC_{50} (TZP)	MIC range (AMK)	MIC_{50} (AMK)	MIC range (CIP)	MIC_{50} (CIP)
E. cloacae (54)	8->512	256	8->256	128	64->256	256	0.25-32	16	<0.25-32	0.5	2->512	64	1->512	256	0.06->32	16
S. marcescens (5)	8->512	256	8->256	128	64->256	256	0.25-32	16	<0.25-32	0.5	2->512	64	1->512	256	0.06->32	16
C. freundii (4)	8->512	256	8->256	128	64->256	256	0.25-32	16	<0.25-32	0.5	2->512	64	1->512	256	0.06->32	16
E.coli J53 AZ^R	0.25	-	0.25	-	0.5	-	<0.25	-	<0.25	-	0.25	-	0.25		0.01	-
T-*E. cloacae*[1] (46)	0.5-512	128	0.5-256	64	4-256	256	<0.25-32	16	<0.25-16	0.5	0.5-512	32	0.5->512	256	0.06-0.5	0.25
T-*S. marcescens* (5)	0.5-512	128	0.5-256	64	4-256	256	<0.25-32	16	<0.25-16	0.5	0.5-512	32	0.5->512	256	0.06-0.5	0.25
T-*C. freundii* (2)	0.5-512	128	0.5-256	64	4-256	256	<0.25-32	16	<0.25-16	0.5	0.5-512	32	0.5->512	256	0.06-0.5	0.25

1. T-*E. cloacae* was transconjugant from *E. cloacae*.

Table 2. The genotypes of pAmpC producers and their transconjugants

genotypes	p*E. cloacae* (54)[1]	p*S. marcescens* (5)	p*C. freundii* (4)	T-*E. cloacae* (51)[2]	T-*S. marcescens* (5)	T-*C. freundii* (4)
DHA-1	32	4	3	30	4	3
ACT-1	22	1	1	21	1	1

1. p*E. cloacae* was pAmpCs producers from *E. cloacae*.
2. T-*E. cloacae* was transconjugant from *E. cloacae*.

Table 3. Distribution of other β-lactamases among 23 pAmpC *E. cloacae* and 19 transconjugants from pAmpC *E. cloacae*

pAmpC producers	genotypes	transconjugants from
No.3	CTX-M-14/DHA-1	No.3
No.15	OXA-1/DHA-1	No.15
No.22	TEM-1/ACT-1	-[a]
No.29	SHV-5+CTX-M-14/DHA-1	No.29
No.65	TEM-1/DHA-1	No.65
No.78	CTX-M-14/ACT-1	-[a]
No.109	TEM-1+CTX-M-14/ACT-1	No.109
No.134	TEM-1+CTX-M-14/DHA-1	No.134
No.166	CTX-M-15/ACT-1	No.166
No.195	SHV-5/DHA-1	No.195
No.211	SHV-5+CTX-M-14/DHA-1	No.211
No.252	CTX-M-14/ACT-1	No.252
No.284	TEM-1+CTX-M-14/DHA-1	No.284
No.302	TEM-1+SHV-5/DHA-1	-[a]
No.315	CTX-M-14/ACT-1	No.315
No.336	SHV-5+CTX-M-14+OXA-1/DHA-1	No.336
No.362	TEM-1+CTX-M-14/DHA-1	No.362
No.390	CTX-M-15/DHA-1	No.390
No.413	TEM-1+CTX-M-14+OXA-1/DHA-1	No.413
No.445	CTX-M-15/DHA-1	No.445
No.491	SHV-5+CTX-M-14/DHA-1	No.491
No.502	SHV-5+CTX-M-14/DHA-1	No.502
No.527	TEM-1+SHV-5/ACT-1	-[a]

a Conjugation was not performed successfully.