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Identification of Genotypes of Plasmid-Encoded AmpC β-Lactamases From Clinical Isolates and Characterization of Mutations in Their Promoter and Attenuator Regions

GUI-LING LI,* LI-BO DUO,* YING LUAN,* CHENG-YING WANG,†
WEI-PING WANG,† HE-GUANG ZHANG,* QI SUN,* AND GUI-YUN QI*

**Department of Medicine Laboratory, Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China*
†Medicine Laboratory, Department of Urology Surgery, DaQing Oilfield General Hospital, DaQing, Heilongjiang, China

We investigated the occurrence of AmpC β-lactamases among *Escherichia coli* and *Klebsiella pneumoniae* isolates and determined the genotype of plasmid-mediated AmpC β-lactamases at a medical center. The AmpC β-lactamase promoter and attenuator were amplified from chromosomal DNA of high AmpC-producing *E. coli* isolates and sequenced. Antibiotic screening and 3D extract tests showed the presence of AmpC β-lactamase in 3.56% of *K. pneumoniae* and 1.88% of *E. coli* isolates. Ten isolates (six *K. pneumoniae* and four *E. coli*) were positive for extended spectrum β-lactamase (ESBL) as indicated by the double disc diffusion method. DHA-1 plasmid-encoded AmpC β-lactamase was present in 10 *K. pneumoniae* isolates and four *E.coli* isolates. *E. coli* chromosomal AmpC β-lactamase carried polymorphisms in the −42, −32, and −18 bases of the promoter and in the +26 and +27 bases of the attenuator, which may play a role in antibiotic resistance. The observed mutations may have clinical implications for the management of antibiotic-resistant infections.

Key words: *Klebsiella pneumoniae*; *Escherichia coli*; AmpC β-lactamases; DHA-1; Inducible

INTRODUCTION

The synthesis of β-lactamases by Gram-negative bacteria is an important mechanism by which they acquire resistance to β-lactam antibiotics (28). This is a major clinical concern because 1) *E. coli* and *K. pneumoniae* are the most common cause of nosocomial infections and 2) treatment with β-lactam antibiotics represents the major approach to treat infections caused by Gram-negative bacilli (28,30). AmpC β-lactamases are cephalosporinases that hydrolyze cephalosporins and confer resistance to clavulanic acid, β-lactam drugs such as cefoxitin, ceftazidime, most penicillins, and β-lactam–β-lactamase inhibitor combinations (8,23,36). AmpC β-lactamase is inducible in a number of pathogens including *Enterobacter cloacae*, *Citrobacter freundii*, and *Pseudomonas aeruginosa* (22).

Variant cephalosporinases with expanded hydrolytic activity (extended spectrum β-lactamases or ESBLs) have recently emerged as the major source of antibiotic resistance (7,11,27). These ESBLs have been shown to be structurally related to the wild-type cephalosporinases and have an expanded hydrolytic spectrum due to insertions, deletions, or substitutions in the gene (11,27).

The AmpC β-lactamase gene has been reported in chromosomal locations in *Enterobacter*, *Shigella*,

Address correspondence to Li-Bo Duo, Department of Medicine Laboratory, Second Affiliated Hospital of Harbin Medical University, 148 Baojian Road, Harbin, Heilongjiang, 150086, China. Tel: +86-0451-86605363; Fax: +86-0451-86296362; E-mail: duolibo@163.com

Morganella morganii, and *E. coli* (22). AmpC expression in Enterobacteriaceae is upregulated in response to β-lactams via AmpR, a DNA binding protein belonging to the LysR transcriptional regulator family (10). However, since the AmpR gene is not present in *E. coli* and *Shigella* sp., AmpC expression in these organisms is regulated via the AmpC promoter and attenuator sequences (14). Interestingly, the AmpC β-lactamase gene has been shown to be plasmid encoded and highly transmissible in *K. pneumoniae* and *E. coli* (3,13). Plasmid-encoded AmpC β-lactamases are similar to their chromosomal counterparts in conferring resistance to a wide variety of β-lactams (14).

It has been suggested that *K. pneumoniae* does not possess chromosomal AmpC β-lactamase (2). Although AmpC expression in *E. coli* is noninducible or lowly expressed, there are some strains that constitutively overexpress AmpC (29). The tests to phenotypically confirm the presence of AmpC include 1) the disk diffusion test based on comparison of inhibition zone diameters around a cefoxitin wafer; 2) the three-dimensional extract test; and 3) comparison of zone of inhibition in the presence of boronic acid derivatives (AmpC inhibitors) along with β-lactam versus β-lactam alone, while these approaches are not able to differentiate between chromosomal and plasmid-encoded AmpC (11,14). Molecular techniques such as multiplex PCR are emerging as the optimal method to detect, differentiate the different families of AmpC β-lactamase genes, and characterize mutations within the gene (29).

In this study, we investigated the occurrence of AmpC β-lactamases among the different isolates of *E. coli* and *K. pneumoniae*. We used multiplex PCR to determine the genotype of plasmid-encoded AmpC β-lactamases in 21 different AmpC β-lactamase-positive isolates of bacteria. The DNA sequences of AmpC and AmpR derived from clinical isolates were blasted against the GenBank sequence database to gain epidemiological insight. We also evaluated the role of mutations in the promoter and attenuator regions of the *E. coli* AmpC β-lactamase gene in AmpC production. Developing reliable and efficient means of characterizing AmpC β-lactamase-producing pathogens is a valuable step to combat antibiotic-resistant infections.

MATERIALS AND METHODS

Pathogens

A total of 867 bacterial isolates were collected from inpatients and outpatients who were treated at the Second Hospital of Harbin Medical University between 2003 and 2005. Bacterial isolates collected included *K. pneumoniae* ($n = 281$) and *E. coli* ($n = 586$). *E. coli* was collected from a number of infectious sites including urine ($n = 361$), sputum ($n = 118$), secretions including pus, wound secretions, and puncture fluids such as pleural effusions or ascites ($n = 66$), blood ($n = 30$), and others ($n = 11$); *K. pneumoniae* was isolated from urine ($n = 142$), sputum ($n = 109$), secretions ($n = 20$), blood ($n = 6$), and others ($n = 4$). Standard protocols were followed for isolation of bacteria, and all isolates were identified using a WalkAway 40 instrument (Siemens AG, Muenchen, Germany). *E. coli* (ATCC 25922) obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). AmpC-producing strains of *Enterobacter cloacae* 029 M (chromosome-mediated de-repressed continuous high production), *Enterobacter cloacae* 029, and the plasmid-mediated AmpC enzyme (ACT-1)-producing strain *E. coli* DH5α were kind gifts from Prof. Yu in the Department of Infectious Diseases of the First Hospital of Zhejiang Medical University.

Antibiotic Screening Assay

We detected the AmpC phenotype using the Kirby-Bauer antibiotic testing method as recommended by the National Committee for Clinical Laboratory Standards (26). Cefoxitin-resistant bacteria (Cefoxitin FOX, OXOID, UK; 30 μg/tablet) with a zone of inhibition ≤17 mm diameter were classified as suspective AmpC β-lactamase positive. *E. coli* (ATCC25922) sensitive to cefoxitin was used as a negative control. Minimum inhibitory concentration (MIC) was determined using the agar dilution method. The disk diffusion method was employed for the susceptibility testing according to the criteria developed by NCCLS/CLSI. The resistance of AmpC-producing bacteria to 18 common antibiotics was detected, with *E. coli* (ATCC25922) serving as the standard.

Detection of AmpC β-Lactamase and ESBL

AmpC β-lactamase was extracted using the UV sonication method as previously described (8). The supernatants were tested for β-lactamase activity using Nitrocefin-containing wafers and all positive supernatants were sterile filtered and inoculated onto MH plates that were incubated at 35°C for 18–20 h. The three-dimensional extract test with cefoxitin was performed to detect the existence of the AmpC β-lactamase as previously described (8). *E. cloacae* 029M served as a positive control and *E. coli* ATCC25922 as a negative control. Two pairs of discs

(cefotaxime and cefotaxime + clavulanic acid; ceftazidime and ceftazidime + clavulanic acid) were used to confirm ESBL based on CLSI criteria. *K. pneumoniae* ATCC700603 served as a positive control and *E. coli* ATCC25922 as a negative control.

Identification of AmpC Genotype by Multiplex PCR

Multiplex PCR assay and the reported primers for MOXM, CITM, DHAM, ACCM, EBCM, and FOXM were utilized to screen for the AmpC gene and to identify genotypes, as previously described (29). *E. coli* DH5α containing the ACT-1 plasmid encoding AmpC β-lactamase served as positive control. *E. coli* ATCC 25922 served as a negative control. The specificity of the method was tested by performing multiplex PCR on genomic DNA of *E. cloacae* 029M (chromosome encoded AmpC β-lactamase). All PCR reactions were performed on a My Cycler™ PCR instrument (Bio-Rad, Hercules, CA, USA). PCR products were analyzed on 1.5% agarose gels and image analysis was performed on the GIS 2010 Tanon Digital image processing system (Shanghai Tiangen Technology Co., Ltd. China). Bidirectional sequencing of PCR products was performed using the ABI PRISM 377DNA sequencer at Shanghai Sangon Biotech (Shanghai, China).

Cloning of Full-Length AmpC β-Lactamase Gene

The DHA-1 AmpC β-lactamase gene was cloned using primers P1: 5′-GGTTGAATTCGATGAAAA AATCGTTATCTGCAAC-3′ and P2: 5′-GGGGCT GCAGCAGGGAAAAAATTATTCCAGTGC-3′. The DHA-1 AmpR β-lactamase gene was cloned using primers P3: 5′-AGAAGGATCCCAGGTGGATTAT GGTCAGACG-3′ and P4: 5′-GGTAAAGCTTCTG GAAGGTGAGTGAGTTTACG-3′. Primers P1–P4 were designed based on the *M. morganii* sequence (GenBank: AF055067). A DNA fragment, consisting of the −10 and −35 regions in the promoter, attenuator region and partial AmpC structural gene, was cloned using primers P5: 5′-GATCGTTCTGCCGC TGTG-3′ and P6: 5′-GGGCAGCAAATGTGGAG CAA-3′, which were designed based on the *E. coli* K12 DNA sequence (GenBank: AE000487). All primers were designed using the Primer 5 software and synthesized by Shanghai Sangon Biotech. Figure 1 is a schematic showing the relative locations of PCR primers (P1–P4) on the AmpR and AmpC genes. Plasmid isolation was performed using the UNIQ-10 Small-Scale Plasmid Extraction Kit (Shanghai Sangon, Biotech), according to the manufacturer's instructions.

RESULTS

Detection of AmpC β-Lactamase Phenotype

Of the 867 bacterial isolates tested, 21 suspective isolates (2.42%) had a diameter of zone of inhibition of ≤17 mm with the cefoxitin wafers. The nonsusceptible isolates included 10 of the 281 *K. pneumoniae* isolates (3.56%) and 11 of the 586 *E. coli* isolates (1.88%). We used a zone of inhibition diameter of ≤17 mm based on a previous report (8), which showed that using a zone of inhibition diameter of ≤14 mm would have resulted in failure of detect some AmpC-producing isolates. Disk diffusion assays showed that >70% of the bacteria were resistant to third-generation cephalosporins; 9.5% (1/21) were resistant to piperacillin/tazobactam; 33.3% (7/21) were resistant to cefoperazone/sulbactam; 52.4% (11/21) were resistant to amikacin; 81% (17/21) were resistant to gentamicin; 42.9% (9/21) were resistant to cefepime; and 90.5% were resistant to levofloxacin. Tienam-resistant bacteria were not observed.

Three-dimensional extract tests showed that all 21 isolates with a diameter of zone of inhibition of ≤17 mm were likely to be positive for AmpC β-lactamase, while only six *K. pneumoniae* isolates and four *E. coli* isolates were positive for ESBL, as indicated by the double disc diffusion method.

Multiplex PCR Screening of Plasmid Encoded AmpC β-Lactamase

Expression of plasmid-encoded AmpC β-lactamase was evaluated using multiplex PCR on plasmid DNA extracted from the 10 isolates of *K. pneumoniae*, which were not susceptible to cefoxitin and were positive for AmpC β-lactamase. All 10 isolates showed the presence of a 405-bp band, which was 100% homologous to the AmpC gene of *M. morganii* (DHA-1 AmpC β-lactamase; GenBank: AF055067). We performed multiplex PCR on plasmid DNA extracted from the 11 isolates of *E. coli*, which were not susceptible to cefoxitin and were positive for AmpC β-lactamase. Of these, four isolates showed the presence of a 405-bp band, which was 100% homologous to the AmpC gene of *M. morganii* (DHA-1 AmpC β-lactamase).

Sequence Analyses of K. pneumoniae *and* E. coli *Plasmid Encoded AmpC β-Lactamase*

We used primers P1 and P2 in order to compare the sequences of the full-length DHA-1 plasmid-encoded AmpC β-lactamase gene from different isolates of *K. pneumoniae*. Sequencing of the 1140-bp full-length DHA-1 AmpC β-lactamase gene PCR

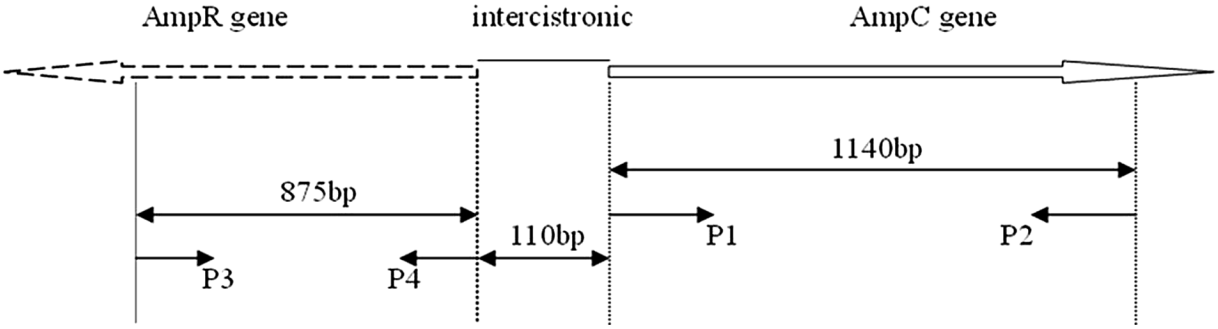

Figure 1. A schematic showing the relative locations of PCR primers (P1–P4) on the AmpR and AmpC genes.

product indicated the presence of polymorphisms in *ampC* gene in all 10 *K. pneumoniae* isolates compared with the DHA-1 AmpC β-lactamase gene of *M. morganii* (Table 1). Although most of the mutations were silent and did not cause an amino acid alteration, the 311T → C in the Kp17 isolate resulted in Leu → Pro (Table 1). Kp17 was cetoxifin resistant and was ESBL positive, suggesting a complex mechanism of resistance. The homology compared to *M. morganii* was as high as 99.1%. Our sequencing data also showed a 100% homology compared to *K. pneumoniae* (AmpC structural gene: AY585202). The AmpC gene sequence in 7 of the 10 the *K. pneumoniae* isolates were homologous and designated as Kp1 and deposited in GenBank (DQ223022). Kp1 was resistant to cetoxifin and was negative for ESBL activity. Table 2 shows the results of a BLAST search using DQ223022 against isolates from different countries.

Primers P1 and P2 successfully cloned the DHA-1 plasmid-encoded AmpC β-lactamase gene from 4 out of 11 *E. coli* isolates, resulting in a band at approximately 1140 bp.

Isolation and Sequence Analysis of the K. pneumoniae *and* E. coli *AmpC* β*-Lactamase Regulatory (AmpR) Genes*

The 875-bp full-length DHA-1 β-lactamase gene regulatory gene (AmpR), encoding a protein of 292 amino acids, was PCR amplified using primers P3 and P4 from the extracted plasmid DNA. Sequencing revealed the presence of polymorphisms in *ampR* gene in all 10 isolates compared with the DHA-1 AmpR β-lactamase gene of *M. morganii* (Table 3). Most mutations were silent except that 215T → C in the isolate of Kp17 caused the switch of Val → Ala. Our sequencing data showed a 98.7% ribonucleotide homology, a 99.9% amino acid homology compared with DHA-1 AmpR gene of *M. morganii*. Our PCR product also showed a 99.5% ribonucleotide homology with the AmpR gene of *K. pneumoniae* (Gen

TABLE 1
ampC SNP RESULTS OF 10 *Klebsiella pneumoniae* ISOLATES PRODUCING DHA-1 AmpC β-LACTAMASE

	Nucleotide Change												Amino Acid Change
	184	186	189	190	311	819	821	915	945	1005	1032	1110	104
Kp1		C→T				T→C	A→C	C→T	C→T	G→C	G→C	C→T	
Kp4		C→T				T→C	A→C	C→T	C→T	G→C	G→C	C→T	
Kp11		C→T		T→A		T→C	A→C	C→T	C→T	G→C	G→C	C→T	
Kp13		C→T				T→C	A→C	C→T	C→T	G→C	G→C	C→T	
Kp17		C→T			T→C	T→C	A→C	C→T	C→T	G→C	G→C	C→T	Leu→Pro
Kp18		C→T				T→C	A→C	C→T	C→T	G→C	G→C	C→T	
Kp19		C→T				T→C	A→C	C→T	C→T	G→C	G→C	C→T	
Kp20	T→A	C→T				T→C	A→C	C→T	C→T	G→C	G→C	C→T	
Kp21	T→A	C→T				T→C	A→C	C→T	C→T	G→C	G→C	C→T	
Kp22		C→T	T→G			T→C	A→C	C→T	C→T	G→C	G→C	C→T	

Compared with *Morganella morganii ampC* (GenBank No. AF055067).

TABLE 2
BLAST ANALYSIS OF DHA-1 AmpC β-LACTAMASE (GenBank No. DQ223022)

Country	GenBank No.	Nucleotide Homogeneity (%)	Amino Acid Homogeneity (%)	Reference
Zhejiang, China	AY705809	100%	100%	34
Korea	AY205600	100%	100%	35
Paris, France	AJ971345	100%	100%	32
Singapore	EF406115	100%	100%	20
Japan	AY887124	100%	100%	25

Bank: AY887124). The AmpR gene sequence, which was from the Kp1 isolate, was deposited in GenBank (EU476911). Table 4 shows the results of a BLAST search using EU476911 against isolates from different countries.

The AmpR gene was successfully cloned using primers P3 and P4 in 4 out of 11 *E. coli* isolates, resulting in a band at approximately 875 bp.

PCR Amplification of the AmpC β*-Lactamase Promoter and Attenuator From* E. coli *Chromosomal DNA*

Primers P5 and P6 were used to amplify a DNA segment containing the promoter and attenuator regions of the AmpC β-lactamase gene in seven isolates of *E. coli* overexpressing nonplasmid-encoded AmpC β-lactamase, which were negative for plasmid-encoded AmpC β-lactamase by multiplex PCR screening. A 271-bp band was amplified in six of these isolates and sequenced, while no band was amplified from the seventh isolate. Comparison of these sequences with *E. coli* K12 showed a number of mutations in the −88 to +58 region (Table 5).

Based on our results, we classified the polymorphisms in the *E. coli* AmpC gene into three genotypes. 1) Genotype A: Four *E. coli* isolates (*E. coli* 9, *E. coli* 10, *E. coli* 51, and *E. coli* 52) carried mutations of the −42 and −18 bases to form a new promoter. The −42C → T and −18G → A polymorphisms resulted in the formation of a new −35 region (TTGACA) upstream to the original −35 region. The alteration of −18G → A resulted in the formation of a new −10 region (TATCGT) upstream to the original −10 region. These changes resulted in the formation of a new gene cassette in which the original conserved −35 region was identical to resulting potent promoter. The 17-bp spacing between the −35 and −10 regions resulted in AmpC β-lactamase overexpression in these four isolates, with a cefoxitin minimum inhibitory concentration (MIC) of 128 μg/ml. Additionally, these four *E. coli* isolates also carried mutations at the −88, −82, −1, and +58 bases. 2) Genotype B: One *E. coli* isolate (*E. coli* 7) carried a mutation in the fourth base of the conserved −35 region (−32T → A), which altered the original TTG TCA to TTGACA and created a potent promoter to drive AmpC β-lactamase overexpression. The cefoxitin

TABLE 3
ampR SNP RESULTS OF 10 *Klebsiella pneumoniae* ISOLATES PRODUCING DHA-1 AmpC β-LACTAMASE

	Nucleotide Change													Amino Acid Change
	75	129	137	171	177	180	215	340	348	381	393	396	405	72
Kp1	T→C	T→G		A→G	A→G			A→G	T→C	T→C	T→C	C→T	T→C	
Kp4	T→C	T→G		A→G	A→G			A→G	T→C	T→C	T→C	C→T	T→C	
Kp11	T→C	T→G	A→G	A→G	A→G			A→G	T→C	T→C	T→C	C→T	T→C	
Kp13	T→C	T→G		A→G	A→G			A→G	T→C	T→C	T→C	C→T	T→C	
Kp17	T→C	T→G		A→G	A→G		T→C	A→G	T→C	T→C	T→C	C→T	T→C	Val→Ala
Kp18	T→C	T→G		A→G	A→G			A→G	T→C	T→C	T→C	C→T	T→C	
Kp19	T→C	T→G		A→G	A→G	G→A		A→G	T→C	T→C	T→C	C→T	T→C	
Kp20	T→C	T→G		A→G	A→G			A→G	T→C	T→C	T→C	C→T	T→C	
Kp21	T→C	T→G		A→G	A→G			A→G	T→C	T→C	T→C	C→T	T→C	
Kp22	T→C	T→G		A→G	A→G			A→G	T→C	T→C	T→C	C→T	T→C	

Compared with *Morganella morganii ampR* (GenBank No. AF055067).

TABLE 4
BLAST ANALYSIS OF AmpR (GenBank No. EU476911)

Country	GenBank No.	Nucleotide Change	Nucleotide Homogeneity (%)	Reference
Zhejiang, China	AY705809	No	100%	34
Paris, France	AJ971345	7786A→C	99%	32
		210T→P	99%	
Japan	AY887124	No	100%	25

MIC was 128 μg/ml. Our sequencing results also showed that this promoter had mutations of −76, −1, and +58 bases. 3) Genotype C: One *E. coli* isolate (*E. coli* B14) carried mutations in the structural gene of attenuator. Substitution of four bases in the attenuator (+22C → T; +26, 27TA → GT; +32G → A) resulted in AmpC β-lactamase overexpression with a high cefoxitin MIC (512 μg/ml).

DISCUSSION

In this study, we identified plasmid-encoded AmpC β-lactamase in the *E. coli* and *K. pneumoniae* isolated in our clinical practice. We used antibiotic screening and the three-dimensional extract test to show the presence of plasmid-encoded AmpC β-lactamase in 3.56% (10/81) of the *K. pneumoniae* isolates and 1.88% (11/586) of the *E. coli* isolates at our hospital. In addition, 10 isolates (six *K. pneumoniae* and four *E. coli*) also produced ESBLs. Our PCR analysis confirmed the presence of CTX-M-1 and CTX-M-9 ESBLs (data not shown). We used multiplex PCR to determine the genotypes of plasmid-encoded AmpC β-lactamase. Of the 11 isolates of *E. coli* that were not susceptible to cefoxitin, only four isolates were positive for plasmid-encoded AmpC β-lactamase by multiplex PCR analysis. Although the remaining seven (three from urine, one from blood, two from sputum, and one from secretions at incision site) were positive for AmpC β-lactamase using the three-dimensional extract test, they were negative by multiplex PCR analysis. Our data are explained by the presence of polymorphisms at the AmpC promoter and/or attenuator regions in six of these *E. coli* isolates, resulting in overexpression of nonplasmid AmpC β-lactamase.

AmpC β-lactamase is traditionally identified using cefoxitin screening and the three-dimensional extract test (14), and a combination of the two methods accurately identified plasmid-encoded AmpC β-lactamase in *K. pneumoniae* where chromosomal AmpC is absent (8). However, in bacteria such as *Citrobacter freundii*, which express chromosomal AmpC, cefoxitin screening combined with the three-dimensional extract test cannot differentiate chromosomal from plasmid-encoded AmpC enzyme. *E. coli* carries the chromosomal AmpC gene, but expresses low levels of AmpC because it is deficient in AmpR. *E. coli* is therefore usually sensitive to cefoxitin, and negative in the three-dimensional extract test. Cefoxitin resistance in this situation could indicate 1) the acquisition of a plasmid-encoded AmpC gene, which would confer a positive result in cefoxitin screening combined with the three-dimensional extract test or 2) presence of a mutation at the promoter/attenuator of the chromosomal AmpC gene, which would confer cefoxitin resistantance (24). In this study, we used a combination of antibiotic screening and the three-dimensional extract test, followed by multiplex PCR to identify genotypes of plasmid-encoded AmpC β-lactamases isolated from our hospital and to characterize mutations in the promoter and attenuator regions of chromosomal AmpC β-lactamase.

TABLE 5
MUTATIONS IN THE PROMOTER AND ATTENUATOR OF AmpC β-LACTAMASE IN CHROMOSOMAL DNA of *E. coli* ISOLATES

	Mutations at Promoter and Attenuator	No. of Mutated Bases	MIC (μg/ml)	GenBank Accession No.
E. coli 7	−76G→A, −32T→A, −1C→T, +58C→T	4	128	DQ263691
E. coli 9	−88C→T, −82A→G, −79T→C, −42C→T, −18G→A, −1C→T, +58C→T	7	128	DQ263690
E. coli 10	88C→T, −82A→G, −42C→T, −18G→A, −1C→T, +58C→T	6	128	DQ263692
E. coli 51	−88C→T, −82A→G, −80C→T, −42C→T, −18G→A, −1C→T, +58C→T	7	128	DQ263693
E. coli 52	−88C→T, −82A→G, −42C→T, −18G→A, −1C→T, +58C→T	6	128	DQ263692
E. coli B14	−76G→A, +22C→T, +26,27TA→GT, +32G→A, +58C→T	6	512	DQ263694

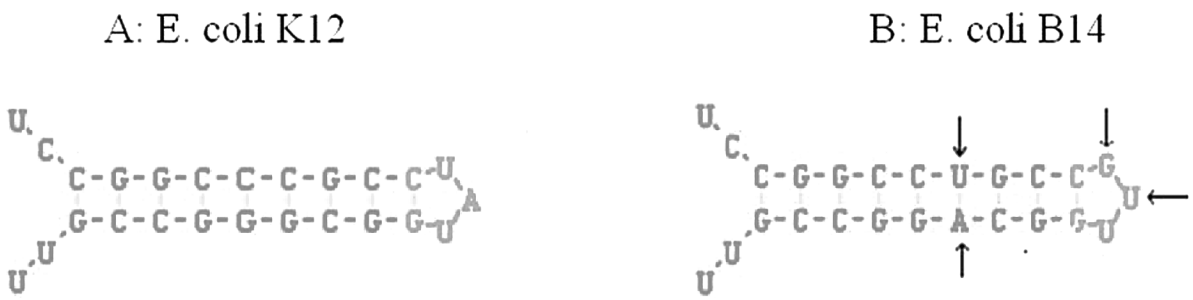

Figure 2. The secondary structure of *E. coli* AmpC β-lactamase attenuator RNA in *E. coli* K12 (A) and *E. coli* B14 (B).

The AmpC β-lactamase PCR products from our *K. pneumoniae* and *E. coli* isolates were 100% homologous to DHA-1 AmpC β-lactamase of *M. morganii*, which is one of the two major genotypes found in China (6,17,33). It would be interesting to investigate if regional differences in the use of antibiotics dictate differences in the distribution of the different genotypes. The inducibility of DHA-1 plasmid-encoded AmpC β-lactamase makes it important to exercise caution in the use of third-generation cephalosporins, β-lactamase inhibitors, and cephamycins or even carbapenems in clinical practice.

The DHA-1 plasmid-encoded AmpC β-lactamase gene is characterized by the presence of the inducible AmpR gene upstream to the AmpC β-lactamase gene (1,21). AmpR, which belongs to the LysR family of transcription factors, binds a 38-bp sequence upstream of the AmpC gene via a helix–turn–helix motif at its N-terminal region in order to initiate transcription in the presence of an inducer (10). Mutation of AmpR gene was previously shown to result in constitutive overexpression of AmpC β-lactamase (21). AmpR has also been shown to play a role in the pathogenesis of *Pseudomonas aeroginosa* and *K. pneumoniae* (12). We showed the presence of a plasmid-encoded AmpR gene in 10 *K. pneumoniae* isolates and in four *E. coli* isolates, which might play a role in regulation of the AmpC β-lactamase gene.

Increased AmpC β-lactamase expression in *E. coli* could be due to 1) mutations in the AmpC promoter region leading to increased transcription efficiency; 2) mutation of structural gene of the attenuator, resulting in alterations in the hairpin structures, which then lead to attenuation of transcriptional inhibition; 3) insertion of a sequence in the promoter region, which may lead to formation of a more potent promoter; 4) increase in the number of copies of the AmpC β-lactamase gene (16). In the present study, our sequencing data ruled out the latter two possibilities.

Mutation of the −42 and −32 regions of the *E. coli* AmpC β-lactamase was previously reported to play an important role in AmpC β-lactamase overexpression and 91.8% of the AmpC promoters in resistant strains carried the −42C → T mutation (4,5,19). These data were consistent with our results which showed the presence of this mutation in 66.7% (4/6) of *E. coli* isolates, which overexpressed AmpC β-lactamase. We showed that bacteria with mutations of the −42 or −32 bases had a higher cefoxitin MIC compared to wild-type *E. coli* (218 μg/ml vs. 0.5–4 μg/ml).

A 17 base region between the −35 and −10 regions was previously shown to constitute an ideal promoter region with a 10–15-fold higher transcription efficiency compared to a promoter containing 16 bases or 18 bases (31). Our findings demonstrated that the −42C → T mutation resulted in the formation of a new −35 region and the mutation of −18G → A formed a new −10 region in four of the *E. coli* genotype A isolates. However, since the segment between the two new regions included 17 bases, the transcription activity of these isolates was comparable to that of *E. coli* genotype B, which had a mutation of the −35 potent promoter region and the cefoxitin MIC was still 128 μg/ml.

The attenuator of *E. coli* K12 has a typical hairpin structure that inhibits transcription (9,15) (Fig. 2A). We showed the presence of four mutations in the attenuator region of *E. coli* B14 (+22C → T; +26T → G, +27A → T; +32G → A), resulting in a change in the hairpin structure to an asymmetrical G-U-U structure (Fig. 2B). We suggest that the resultant relief of transcriptional inhibition leads to AmpC β-lactamase overexpression, and a high cefoxitin MIC value (512 μg/ml). Our data are in agreement with a previous study showing the role of the attenuator and attenuator mutations on the expression of AmpC β-lactamase and increased cefoxitin MIC values (18).

In summary, we identified plasmid-encoded AmpC β-lactamase in *E. coli* and *K. pneumoniae* isolates from our hospital. We genotyped the different non-plasmid encoded AmpC β-lactamases and identified polymorphisms in the −42, −32, and −18 bases of the *E. coli* AmpC β-lactamase promoter and in the +26 and +27 bases in the attenuator. The mutations in the

promoter and attenuator regions might result in overexpression of nonplasmid AmpC β-lactamase and might play a role in the resistance of these isolates to a number of antibiotics. Our data have important clinical implications for the management of antibiotic-resistant infections.

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