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Prevalence of AmpC and Extended-Spectrum Beta-Lactamase Genes in *Klebsiella pneumoniae* and *Escherichia coli* Isolates

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Abstract

Background: In recent years, the prevalence of antibiotic resistance has steadily increased and also the antibiotic-resistant strains producing extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases have emerged among the Enterobacteriaceae, predominantly in *Escherichia coli* and *Klebsiella pneumoniae* species.
Objectives: This prospective study aimed at determining the production of ESBL or AmpC, phenotypically and also at the molecular level, in *E. coli* and *K. pneumoniae* isolates collected from various clinical specimens.
Methods: In total, 78 *K. pneumoniae* and 92 *E. coli* isolates were collected from various clinical infectious sources available in different wards of the Imam Reza Hospital, Tabriz, Iran, from July 2017 to December 2018. All isolates were subjected to antimicrobial susceptibility testing. MAST 4-disc test and polymerase chain reaction (PCR) were applied for phenotypic and genotypic detection of ESBLs and plasmid-encoded AmpCs (pAmpCs) among isolates, respectively.
Results: Overall, 78 *K. pneumoniae* and 92 *E. coli* isolates were evaluated, of which 46 *K. pneumoniae* (58.9%) and 51 *E. coli* (55.4%) isolates were resistant to cefotaxime/ceftazidime and included in the study. Among the *K. pneumoniae* and *E. coli* isolates resistant to cefotaxime/ceftazidime, 40 (86.9%) and 40 (78.4%) isolates were ESBL producers and 8 (17.3%) and 2 (3.9%) isolates were pAmpC producers, respectively. In addition, 40 *E. coli* (78.4%) isolates were positive for both *CTX-M-14* and *CTX-M-15* genes. Regarding *K. pneumoniae* isolates, 40 isolates (86.9%) were positive for *CTX-M-15* gene and 18 isolates (39.1%) for *CTX-M-14* gene. Among 51 ceftazidime/cefotaxime-resistant *E. coli* isolates, 32 isolates (62.7%) were positive for *DHA-1* gene and 33 isolates (64.7%) isolates for *CMY-2* gene. Also, among 46 ceftazidime/cefotaxime-resistant *K. pneumoniae* isolates, 15 isolates (32.6%) had *DHA-1* gene and 27 isolates (58.7%) had *CMY-2* gene in the genome.
Conclusions: The high prevalence of ESBL and AmpC production among *E. coil* and *K. pneumoniae* isolates was a serious concern in the studied region. Therefore, a simple and rapid PCR-based technique is essential to detect and distinguish various pAmpC and ESBL genes to discriminate other resistance determinants.

Keywords: *Escherichia coli*, *Klebsiella pneumoniae*, Gen, ESBL, AmpC β-lactamases, *CTX-M-1*

1. Background

Escherichia coli and *Klebsiella pneumoniae* have been one of the major causes of nosocomial and community-acquired infections leading to primary bacteremia, urinary tract infection, and intra-abdominal infection (1, 2). In recent years, the prevalence of antibiotic resistance has steadily increased and also antibiotic-resistant strains with AmpC beta-lactamases (AmpCs) and extended-spectrum beta-lactamases (ESBLs) (3, 4) have emerged among the Enterobacteriaceae isolates, predominantly in *K. pneumoniae* and *E. coli* species (5, 6), of which ESBLs and AmpCs are of great concern. AmpCs are clinically important cephalosporinases encoded on the chromosomes of several species of Enterobacteriaceae. ESBLs are derivatives of the common beta-lactamases that have undergone one or more amino acid substitutions near the active site of the enzyme, leading to an increase in their affinity and the hydrolytic activity against third-generation cephalosporins and monobactams. The extensive use of new-generation cephalosporins has been a strong factor to assess newer b-lactamases. As these enzymes (b-lactamase) are coded by transferable conjugative plasmids, resistance to other antibiotics, such as aminoglycosides has been a concern (7). Although the distribution of CTX-M-producing strains seems to be limited to the particular areas of the world

during the 1990s, it has changed during the past decade. Recent epidemiological investigations on ESBL-producing strains have confirmed a dramatic increase in the prevalence of CTX-M enzymes (8). In species that do not naturally produce AmpC, the genes are also encoded on plasmids (9). In addition, pAmpC can be grouped into seven families: ACC (ambler class C-1), CMY, DHA (10), FOX, MIR, ACT, and MOX. These enzymes have been found worldwide, with CMY-2 being the most prevalent (10, 11). Most ESBLs can be grouped into three families: CTX-M, SHV, and TEM types (12, 13). Recent studies on the prevalence of genotypic and phenotypic detection of ESBL and AmpC beta-lactamase in the northwest of Iran were conducted in 2015 and 2016 (14, 15). Therefore, the results of this study can indicate the prevalence rate of ESBL and AmpC beta-lactamase over the past two years until 2018, which is helpful for the treatment and prevention process of such isolates.

2. Objectives

The present study aimed at determining the frequency of genes encoding AmpC beta-lactamase and ESBLs in *K. pneumoniae* and *E. coli* isolates collected from clinical samples available in the Imam Reza Hospital, Tabriz over six months during 2017 - 2018.

3. Methods

3.1. Selection of the Strains

In this prospective study, the considered isolates were selected from two species of Enterobacteriaceae obtained from the Clinical Microbiology Laboratory in Imam Reza Hospital, Tabriz, Iran. From July 2017 to December 2018, 92 consecutive, non-duplicate isolates of *E. coli* were collected from specimens with urinary tract infections and 78 isolates of *K. pneumoniae* were collected from various clinical specimens, including urine, blood, wound, trachea, sputum, and body fluids. Standardized methods and biochemical tests, such as Gram staining, colony morphology, and motility were performed for the identification of the isolates (16). The isolates identified as *K. pneumoniae* and *E. coli* were included the first time. The inclusion criteria were the isolates with resistance at least against one of the third-generation cephalosporins, such as ceftazidime, cefotaxime, and ceftriaxone. This study was approved by the National Ethics Committee on Human Research (approval code: 15730507952019; 2017-04-24).

3.2. Antibiotic Susceptibility Testing

Antimicrobial susceptibility test was completed by the disk diffusion methods according to the Clinical and Laboratory Standards Institute (CLSI; 2017) to determine the susceptibility of the *E. coli* and *K. pneumoniae* strains to the antibiotics on Muller-Hinton agar (17). Antibiotic discs (master amikacin (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), ceftazidime (30 μg), cefotaxime (30 μg), piperacillin-tazobactam (100/10 μg), nitrofurantoin (300 μg), imipenem (5 μg), meropenem (5 μg), cotrimoxazol (25 μg), and colistin (10 μg) were used for antimicrobial susceptibility tests. *E. coli* ATCC 25922 was used as a control strain.

3.3. Phenotypic Detection of the AmpC and ESBL β-Lactamases by D68C AmpC and ESBL Detection Set

D68C test includes cefpodoxime as an agent for the identification of both chromosomal and plasmid-mediated AmpC. This test uses boronic acid as a pAmpC inhibitor and clavulanic acid as an ESBL inhibitor. The detection set consists of four disks: disk A (cefpodoxime 10 μg $\times$ 1), disk B (cefpodoxime 10 μg and ESBL inhibitor $\times$ 1), disk C (cefpodoxime 10 μg and AmpC inhibitor $\times$ 1), and disk D (cefpodoxime 10 μg + ESBL inhibitor + AmpC inhibitor $\times$ 1). The test was carried out and interpreted according to the manufacturer's instructions (MASTDISCS™ID, UK). An isolate is considered as 'only AmpC-positive' when the difference between zone sizes of B-A and D-C was $<$ 5 mm and between D-B and C-A was $\geq$ 5 mm. In an 'only ESBL-positive' isolate, the difference between zone sizes of B-A and D-C was $\geq$ 5 mm and between D-B and C-A was $<$ 4 mm. Regarding both 'AmpC- and ESBL-positive' isolates, the difference between zone sizes of D-C was $\geq$ 5 mm, whereas it was $<$ 4 mm for B-A. In both 'AmpC- and ESBL-negative' isolates, the difference between all zone sizes is $\leq$ 2mm. A bacterial suspension with a density of 0.5 McFarland opacity standards was cultured on Mueller-Hinton agar using a sterile swab. The disks were put on plates followed by incubation at 37°C for 24 h. The relevant results were interpreted by comparing different zones of the four disks (18).

3.4. DNA Extraction and Polymerase Chain Reaction (PCR)

Bacterial colonies from the media culture plate were picked and then suspended in 360 μL milli-Q water followed by boiling at 95°C for 15 min and the suspension was centrifuged about 1 min at 13,000 rpm. The obtained supernatant was collected and kept at -20°C for PCR reactions. The used primers for ESBL and AmpC-producing genes are presented in Table 1. The PCR mixture for the mentioned primers was the same, including a 20 μL of the reaction mixture and 2 μL of the extracted DNA added to the Master PCR mixture (Yekta Tajhiz Azma®, Iran). The used mix for the detection of the considered genes contained 5 pmol of

each primer (Yekta Tajhiz Azma®, Iran). The amplification was done in a DNA thermal cycler (Eppendorf master cycler gradient, Germany). Table 2 presents the PCR condition for each gene. Finally, electrophoresis was used to analyze PCR products in a 1% agarose gel and also UV transillumination was applied to show the bands on the gel.

For identification of the species, *K. pneumoniae* ATCC 700603 and *Escherichia col*i ATCC 25922 (Pasteur Institute, Iran) were used as standard strains.

3.5. Statistical Analysis

SPSS version 20 software was used for statistical analysis (IBM SPSS Statistics, USA). Descriptive statistics and chi-square or Fisher's exact tests were used to assess the presence of ESBL-producing and AmpC-producing genes. P value < 0.05 was considered as statistically significant.

4. Results

Of 289 specimens sent to the Microbiology Laboratory, 92 isolates were identified as *E. coli* and 78 isolates as *K. pneumoniae*. The isolates were tested for susceptibility by the disk diffusion method and 46 *K. pneumoniae* (58.9%) and 51 *E. coli* (55.4%) isolates were resistant to either all three third-generation cephalosporins (CTR, CAZ, and CTX) or none. The ESBL phenotype confirmation was detected by the Mast D68C test. Forty out of 46 isolates of *K. pneumoniae* and 40 out of 51 cephalosporin-resistant isolates of *E. coli* were ESBL producers.

These isolates were detected by the Mast D68C test and 8 *K. pneumoniae* (17.3%) and 2 *E. coli* (3.9%) isolates were AmpC-positive. in addition, 40 ESBL-producing *E. coli* were isolated from 12 male (30%) and 28 female (70%) patients. All ESBL-producing *E. coli* isolates were isolated from the urine. A total of 40 ESBL-producing *K. pneumoniae* isolates were collected from 21 female (52.5%) and 19 male (47.5%) patients. Moreover, 15 isolates (37.5%) of the ESBL-producing *K. pneumoniae* were collected from the urinary tract, 11 isolates (25.5%) from wounds, 8 isolates (20%) from blood, 3 isolates (7.5%) from the trachea, and 3 isolates (7.5%) from body fluids.

The resistance patterns of these ESBL-producing *K. pneumoniae* and *E. coli* isolates against a spectrum of 11 selected antimicrobial agents of different classes were checked (Figure 1). The isolates showed the highest sensitivity against meropenem and imipenem in *K. pneumoniae* and also nitrofurantoin, amikacin, and imipenem in *E. coli*.

All ceftazidime/cefotaxime-resistant isolates were examined by PCR for the presence of *CTX-M-14* and *CTX-M-15* genes. The results indicated that 40 isolates (78.4%) of the *E. coli* were positive for both *CTX-M-14* and *CTX-M-15* genes. In *K. pneumoniae* isolates, 40 isolates (86.9%) were positive for the *CTX-M-15* gene and 18 isolates (39.1%) for the *CTX-M-14* gene. The distribution of *CTX-M-14* and *CTX-M-15* genes among clinical specimens of the *K. pneumoniae* isolates is provided in Appendix 1 in Supplementary File.

Two AmpC-producing genes, including *DHA-1* and *CMY-2* were assessed and detected by the PCR method in all ceftazidime/cefotaxime-resistant isolates. The results showed that of 51 ceftazidime/cefotaxime-resistant *E. coli* isolates, 32 isolates (62.7%) were positive for *DHA-1* gene and 33 isolates (64.7%) for *CMY-2* gene. Also, of 46 ceftazidime/cefotaxime-resistant *K. pneumoniae* isolates, 15 isolates (32.6%) had *DHA-1* gene and 27 isolates (58.7%) had *CMY-2* gene in their genomes. Comparatively, *E. coli* isolates more frequency had both ESBL-producing and AmpC-producing genes than *K. pneumoniae* isolates.

5. Discussion

The prevalence of Gram-negative bacilli, especially ESBLs-producing *K. pneumoniae* and *E. coli* has increased from 10% to 40% in different regions of the world (19). Also, infections caused by ESBL-producing strains that may harbor pAmpC β-lactamase are associated with a higher mortality rate and complicated treatment process with third-generation cephalosporins (10).

High resistance rates were found using the disk diffusion method in our research, which can be related to the experimental usage of such antibiotics in the treatment of clinical infections in our hospitals (20). Therefore, in this investigation, D68C MASTDISCS™ was used for the phenotypic detection of ESBLs and pAmpCs. In the next step, 4 sets of PCR were performed for genotypic detection of the ESBL- and pAmpC-encoding genes. The outbreak of ESBL-producing *K. pneumoniae* and *E. coli* is varied worldwide (21). In addition, the outbreak of ESBL producers in different hospitals is associated with various factors, such as antibiotic policy, the carriage rate among hospital personnel, and the type of disinfection used particularly in the intensive care unit (ICU) ward (4).

In this study, the high prevalence of ESBL among *K. pneumoniae* and *E. coli* isolates in comparison with a low incidence of AmpC β-lactamases can be due to the fact that ESBLs are more common than pAmpCs. We detected β-lactamases genes, including CTX-M-15 and *CTX-M-14* enzymes in the isolates of *E. coli* and *K. pneumoniae*. In the current research, the total incidence of *blaCTX-M-15* among ESBL isolates was 82.6%, which is higher than two previous reports by Sadeghi et al. (15) from Azerbaijan, Iran and Feizabadi et al. (22) from Tehran, Iran. Based on these results, the distribution of this gene has increased

Table 1. The Primers Used for the Detection of Extended-Spectrum Beta-Lactamase (ESBL)-Producing and AmpC-Producing Genes

Target gene	Primer	Sequence (5'-3')	Product Size, bp	Reference
blaDHA-1	DHA-F	AACTTTCACAGGTGTGCTGGGT	405	(5)
	DHA-R	CCGTACGCATACTGGCTTTGC		
blaCMY-2	CMY-2-F	GAT TCC TTG GAC TCT TCAG	1807	(14)
	CMY-2-R	TAA AAC CAG GTT CCC AGA TAG C		
CTX-M-14	CTX-M-14-F	TACCGCAGATAATACGCAGGTG	355	(15)
	CTX-M-14-R	CAGCGTAGGTTCAGTGCGATCC		
CTX-M-15	CTX-M-15-F	GAT TCC TTG GAC TCT TCAG	499	(16)
	CTX-M-15-R	TAA AAC CAG GTT CCC AGA TAG C		

Table 2. Polymerase Chain Reaction Conditions for Extended-Spectrum Beta-Lactamase (ESBL)-Producing (*CTX-M-14* and *CTX-M-15*) and AmpC-Producing Genes (*DHA-1* and *CMY-2*)

Primers	Cycling Condition				
	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
DHA-1	95° for 5 min	95° for 35 s	58° for 35 s	72° for 35 s	72° for 10 min
	Repeated for 30 cycles				
CTX-M-14	95° for 5 min	95° for 35 s	62° for 35 s	72° for 35 s	72° for 10 min
	Repeated for 30 cycles				
CTX-M-15	95° for 5 min	95° for 35s	62° for 35 s	72° for 35 s	72° for 10 min
	Repeated for 30 cycles				
CMY-2	95° for 5 min	95° for 35s	61° for 35 sec	72° for 35 s	72° for 10 min
	Repeated for 38 cycles				

over the past years and it may be one of the most important and common CTX-M β-lactamases in the northwest of Iran. In this study, a total of 78.4% and 39.1% of the cefotaxime/ceftazidime-resistant strains harbored *blaCTX-M-14* in *E. coli* and *K. pneumoniae* isolates and a total of 78.4% and 86.9% of the cefotaxime/ceftazidime-resistant strains harbored *blaCTX-M-15* in *E. coli* and *K. pneumoniae*.

According to the obtained data, all *blaCTX-M-15* and *blaCTX-M-14* strains showed resistance against ceftazidime. *CTX-M-15* enzymes notably demonstrated to have an increased catalytic activity toward ceftazidime, which is consistent with a previous study (23). In addition, *blaCTX-M-15* was the most prevalent gene in our study. The high prevalence of CTX-M among all ESBL-associated enzymes is the main reason for confirming the high mobilization of the ESBL-encoding genes. Barlow et al. realized that *blaCTX-M* genes compared with other class A b-lactamases, are approximately ten times more mobilized to plasmids (24).

Our results are consistent with those of Kim et al. (25) reporting that blaCTX-M-14, and blaCTX-M-15 genes were positive in 7 (22%) and 17 (54%) samples of the *K. pneumoniae* isolates, and in 23 (45%) and 19 (37%) samples of the *E. coli* isolates, respectively. Organisms harboring (AmpCs) are of increasing clinical concern because they show resistance to all beta-lactam drugs, except for carbapenems, cefpirome, and cefepime (9, 26). Unlike ESBLs, they can hydrolyze cephamycins and beta-lactamase inhibitors are not able to inhibit them (9, 26)). AmpC had both chromosomal and plasmid origin in *E. coli* isolates, whereas *K. pneumoniae* had only plasmid-mediated AmpC (9, 26). One of the major challenges for clinical microbiologists is to detect the different types of AmpCs (27). Similar findings have been reported from different parts of India recording the outbreak of AmpC in isolates of Enterobacteriaceae ranging from 2.2% to 20.7% (11, 28). Our findings are in line with this range as 3.9% and 17.39% of the *E. coli* and *K. pneumoniae* isolates were AmpC-positive, respectively. Vandana and Honnavar (29) have assessed the production of AmpC with two methods, including 3-dimensional enzyme test (3DET) and phenylboronic acid disc (PBA). Using the PBA method, 9 (82%) and 24 (58.5%) *K. pneumoniae* and *E. coli* isolates were found AmpC-positive, whereas through the 3DET method, this rate increased to 10 (91%) and 29 (70.7%) isolates, respectively (29). Their obtained results indicated a higher prevalence of AmpC compared with our study. D68C MASTDISCS™ is one of the effective phenotypic meth-

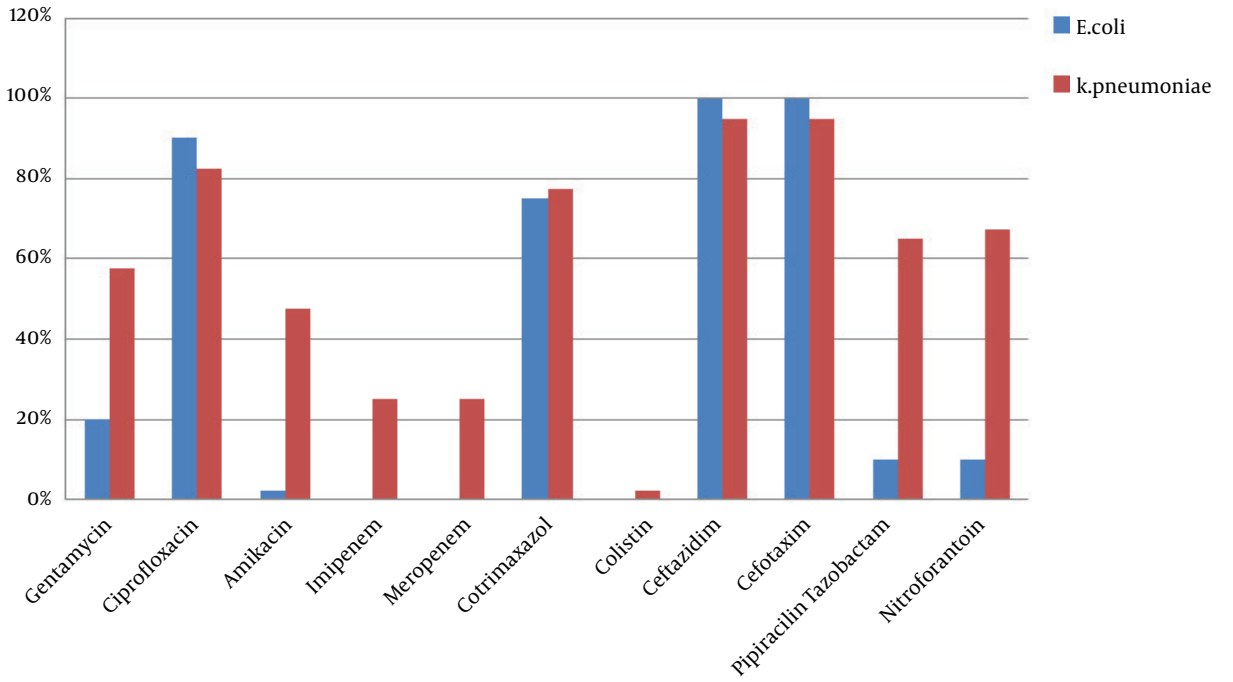

Figure 1. Antimicrobial susceptibility patterns of *E. coli* and *K. pneumoniae* isolates

ods for the detection of ESBL- and/or pAmpC-producing isolates. Considering the report by Ellem et al. (30), our results also confirmed that the specificity of the MAST 4-disc test in the detection of ESBL is higher than its specificity in the detection of AmpC β-lactamase.

Hence, the types of AmpC detection methods influence the reported prevalence rate for AmpC production phenotypically.

This test is able to differentiate MBL and AmpC activity and highlighting the genetic information is needed for the next level. Based on our findings, the plasmid-mediated AmpC genes were found in 39 (76.4%) of *E. coli* and 30 (65.2%) of *K. pneumoniae* isolates. In contrast, both *DHA-1* and *CMY-2* genes were more prevalent in *E. coli* isolates than *K. pneumoniae* isolates in this study.

In Asian countries, especially Korea, Song and et al. indicated that *DHA-1* (66.6%) was the most prevalent type of AmpC β-lactamase followed by *CMY-2* (20%) (6). Unlike, in our research, *CMY-2* was more common than *DHA-1*. Li et al. (31) in China reported that *E. coli* isolates were positive for 52.18% of *CMY-2* and 47.8% of *DHA-1* genes, whereas 96.7% and 3.3% of the *K. pneumoniae* isolates carried *DHA-1* and *CMY-2* genes, respectively. Comparatively, the reported frequencies are lower than our findings. It seems that the extensive and frequent use of broad-spectrum antibiotics can lead to the emergence of resistant isolates in our hospitals. The ICU stay, using invasive procedures, like urinary catheterization, and also exposure to third-generation cephalosporins seem to predispose the patients with such conditions to infections with these resistant Enterobacteriaceae organisms.

5.1. Conclusions

A high prevalence of ESBL-producing strains among *K. pneumoniae* and *E. coli* isolates was found in this investigation. Regarding the epidemiological aspect and therapeutic process, it is important to detect ESBLs in isolates producing both ESBLs and pAmpCs, especially in critical wards, such as ICUs. Hence, more than 60% and 50% of the isolates were positive for ESBL and AmpC genes, respectively. A rapid and simple PCR-based technique is required to detect and distinguish different ESBL and pAmpCs genes to discriminate other resistance determinants.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Authors' Contribution: Study design and writing the manuscript: Alireza Jahantabi. Data analysis and interpretation of the results: Alireza Jahantabi, Farzaneh Hosseini, Mohammad Asgharzadeh, Abbas Akhavan Sepehi, and Hossein Samadi Kafil. Drafting of the manuscript: Alireza Jahantabi and Farzaneh Hosseini. Supervising of the study: Farzaneh Hosseini. All authors revised the final manuscript.

Conflict of Interests: There is no conflict of interests.

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