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Phenotypic and Genotypic Characterization of ESBL-, AmpC-, and Carbapenemase-Producing *Klebsiella pneumoniae* and *Escherichia coli* Isolates

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Significance of the Study

- Given the importance of drug-resistant *Klebsiella pneumoniae* and *Escherichia coli* strains in hospital-acquired infections, the aim of this study was to determine the characteristics of ESBL, AmpC, and carbapenemase-producing isolates. Coexistence of AmpC, ESBL, and carbapenemase genes was observed in some isolates. Implementing up-to-date strategies against such organisms is warranted.

Keywords

Klebsiella pneumoniae · *Escherichia coli* · ESBLs · AmpC · Carbapenemase

Abstract

Objectives: Drug resistance among gram-negative bacteria is a worldwide challenge. Due to the importance of drug-resistant *Klebsiella pneumoniae* and *Escherichia coli* strains in hospital-acquired infections, we aimed to determine the phenotypic and genotypic characteristics of ESBL-, AmpC-, and carbapenemase-producing isolates obtained from hospitalized patients in Tehran and Ilam (Iran). **Materials and**

Methods: In total, 90 *K. pneumoniae* isolates and 65 *E. coli* isolates were collected from various infections. Phenotypic identification of bacterial isolates was performed using standard methods. Phenotypic screening of ESBL, AmpC, and carbapenemase enzymes was carried out. Detection of ESBL, AmpC, and carbapenemase genes was also performed by the PCR method. **Results:** Phenotypic detection tests showed that 36 (40%) *K. pneumoniae* and 23 (35.4%) *E. coli* isolates were ESBL producers. Moreover, 18 (20%) and 6 (9.2%) *K. pneumoniae* and *E. coli* isolates were AmpC producers, respectively. Modified Hodge test results indicated that 39

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(43.3%) *K. pneumoniae* and 18 (27.7%) *E. coli* isolates produced carbapenemase. Molecular tests showed that 40% of *K. pneumoniae* and 36.9% of *E. coli* isolates were ESBL positive. AmpC was detected in 24.4 and 13.8% of *K. pneumoniae* and *E. coli* isolates. Carbapenemase was detected in 34 (37.8%) *K. pneumoniae* and 13 (20%) *E. coli* isolates. **Conclusion:** In this study, 3 *K. pneumoniae* isolates simultaneously carried ESBL, AmpC, and carbapenemase genes. Up-to-date strategies such as combination therapy or utilization of new antimicrobial agents might help to combat such drug-resistant organisms.

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Introduction

Drug resistance in gram-negative bacteria is a worldwide challenge [1]. Major resistance to gram-negative pathogens is related to Enterobacteriaceae, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* [2]. In the Enterobacteriaceae family, *Klebsiella pneumoniae* and *Escherichia coli* have notable drug resistance. Also, they are the common cause of hospital- and community-acquired infections [3, 4].

The wide distribution of extended-spectrum β -lactamases (ESBLs) amongst Enterobacteriaceae has been attributed to the over- or misuse of these antibiotics [5]. ESBLs are able to hydrolyze various types of β -lactam antibiotics, including cephalosporins and monobactams. Therefore, treating infections caused by ESBL-producing bacteria has become a complicated issue [5, 6]. Dissemination of hospital-acquired infections can be prevented via early detection of infections caused by such microorganisms [7].

Carbapenems have been considered as the treatment of choice for ESBL-producing gram-negative bacterial infections. Carbapenemase-producing strains cause serious infections in hospitalized patients and are associated with mortality [8]; hence, the use of molecular techniques could be helpful in accurately diagnosing infections caused by ESBL- and carbapenemase-producing organisms.

Drug-resistant strains of *K. pneumoniae* and *E. coli* in hospital-acquired infections are important [9]; thus, the aim of the present study was to determine the phenotypic and genotypic characterization of ESBL-, AmpC-, and carbapenemase-producing isolates of *K. pneumoniae* and *E. coli* obtained from hospital-associated infections in Tehran and Ilam (Iran).

Materials and Methods

Bacterial Strains

A total of 90 *K. pneumoniae* and 65 *E. coli* isolates were obtained from various infections of hospitalized patients between April 2016 and March 2017 in Tehran and Ilam. Phenotypic and biochemical identification of bacterial isolates was performed according to standard methods [10].

Phenotypic Screening of ESBL, AmpC, and Carbapenemase Enzymes

All isolates were initially screened for ESBL production by a combined disk method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) [11]. Briefly, susceptibility to cefotaxime (30 μ g), cefotaxime/clavulanate (30/10 μ g), ceftazidime (30 μ g), and ceftazidime/clavulanate (30/10 μ g) (Mast Co., UK) was determined on Müller-Hinton agar (Merck Co, Germany). ESBL-producing strains were recognized by an at least 5-mm increase in zone diameter around cefotaxime/clavulanate and ceftazidime/clavulanate disks in comparison with disks without clavulanic acid. *E. coli* ATCC 35218 was used as the control strain [11].

Cefoxitin disks (30 μ g) were used to screen AmpC-producing isolates according to CLSI recommendations [11]. A cefoxitin-cloxacillin double-disk synergy test was carried out to confirm AmpC production, as previously described [12].

The modified Hodge test (MHT) was performed to screen carbapenemase-producing isolates [11]. *K. pneumoniae* ATCC BAA-1705 and BAA-1706 were used as MHT-positive and -negative controls [11].

Detection of ESBL-, AmpC-, and Carbapenemase-Related Genes

Polymerase chain reaction was performed using specific primers to detect genes encoding ESBLs (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{PER}), AmpC (*bla*_{ACC}, *bla*_{DHA}, *bla*_{EBC}, *bla*_{FOX}, *bla*_{MOX}, and *bla*_{CTT}), and carbapenemase (*bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{KPC}, and *bla*_{OXA-48-like}), as previously described [13–17]. The products were separated by electrophoresis in 1% agarose gel with 1 \times TBE (Tris/borate/EDTA) buffer, stained with safe stain load dye (CinnaGen Co., Tehran, Iran) and visualized under ultraviolet illumination.

Statistical Analysis

The distribution of resistance genes among resistant and susceptible isolates was calculated using χ^2 and Fisher's exact tests for each gene. *p* values ≤ 0.05 were considered to be statistically significant.

Results

Phenotypic ESBL detection tests indicated that 36 (40%) *K. pneumoniae* isolates and 23 (35.4%) *E. coli* isolates were ESBL producers. Moreover, 32 (35.5%) *K. pneumoniae* and 19 (29.2%) *E. coli* isolates were AmpC positive, while phenotypic confirmatory tests showed that 18 (20%) and 6 (9.2%) *K. pneumoniae* and *E. coli* strains, respectively, were AmpC producers; 39 (43.3%)

Table 1. Distribution of ESBL-associated genes among *K. pneumoniae* and *E. coli* isolates

Isolate	ESBL		<i>bla</i> _{TEM}	<i>bla</i> _{SHV}	<i>bla</i> _{CTX-M}	<i>bla</i> _{PER}
	phenotypic	genotypic				
<i>K. pneumoniae</i>	36 (40)	36 (40)	21 (23.3)*	19 (21.1)*	10 (11.1)	0
<i>E. coli</i>	23 (35.4)	24 (36.9)	11 (16.9)	11 (16.9)	14 (21.5)*	0

Values are presented as *n* (%). * *p* < 0.05.

Table 2. Distribution of AmpC-related genes among *K. pneumoniae* and *E. coli* isolates

Isolate	AmpC positive			<i>bla</i> _{ACC}	<i>bla</i> _{DHA}	<i>bla</i> _{EBC}	<i>bla</i> _{FOX}	<i>bla</i> _{MOX}	<i>bla</i> _{CIT}
	screening test	confirmatory test	genotypic						
<i>K. pneumoniae</i>	32 (35.5)	18 (20)	22 (24.4)	0	15 (16.6)*	12 (13.3)	0	0	5 (5.5)
<i>E. coli</i>	19 (29.2)	6 (9.2)	9 (13.8)	0	4 (6.1)	3 (4.6)	0	0	2 (3)

Values are presented as *n* (%). * *p* < 0.05.

Table 3. Carbapenem resistance patterns among *K. pneumoniae* and *E. coli* isolates

Isolate	Carbapenemase producer		<i>bla</i> _{IMP}	<i>bla</i> _{VIM}	<i>bla</i> _{NDM}	<i>bla</i> _{KPC}	<i>bla</i> _{OXA-48-like}
	MHT	genotypic					
<i>K. pneumoniae</i>	39 (43.3)	34 (37.8)	1 (1.1)	30 (33.3)*	0	0	13 (14.4)
<i>E. coli</i>	18 (27.7)	13 (20)	0	8 (12.3)	0	0	13 (20)*

Values are presented as *n* (%). MHT, modified Hodge test. * *p* < 0.05.

K. pneumoniae and 18 (27.7%) *E. coli* strains produced carbapenemase.

Molecular methods showed that 36 (40%) *K. pneumoniae* isolates harbored at least 1 of the ESBL-related genes. Distribution of these genes among *K. pneumoniae* strains were *bla*_{TEM} 23.3%, *bla*_{SHV} 21.1%, and *bla*_{CTX-M} 11.1%. Presence of *bla*_{TEM} and *bla*_{SHV} genes in ESBL-producing *K. pneumoniae* isolates was statistically significant (*p* < 0.05). In addition, 24 (36.9%) *E. coli* isolates were ESBL positive. The prevalence of *bla*_{TEM} and *bla*_{SHV} genes was 16.9%, and *bla*_{CTX-M} was observed in 21.5% of isolates. The *bla*_{PER} gene was not detected in any of the strains. The *bla*_{CTX-M} gene was more prevalent in *E. coli* isolates which were ESBL producers (Table 1).

AmpC-associated genes were detected amongst 22 (24.4%) *K. pneumoniae* isolates. *bla*_{DHA}, *bla*_{CIT}, and *bla*_{EBC} were found among 16.6, 5.5, and 13.3% of *K. pneumoniae* strains, respectively. *bla*_{DHA} prevalence in AmpC-posi-

tive *K. pneumoniae* isolates was statistically significant (*p* < 0.05). Furthermore, 9 (13.8%) of the *E. coli* isolates carried AmpC genes, and the distribution percentages of *bla*_{DHA}, *bla*_{CIT}, and *bla*_{EBC} were 6.1, 3, and 4.6%, respectively. All the strains were negative for *bla*_{ACC}, *bla*_{FOX}, and *bla*_{MOX} (Table 2).

Carbapenemase-related genes were detected in 34 (37.8%) *K. pneumoniae* isolates. The prevalence rates of these genes were *bla*_{VIM} 33.3%, *bla*_{OXA-48-like} 14.4%, and *bla*_{IMP} 1.1%. Thirteen (20%) *E. coli* isolates were carbapenemase positive. *bla*_{VIM} and *bla*_{OXA-48-like} were observed in 12.3 and 20% of isolates, respectively. No *bla*_{IMP} gene was detected in *E. coli* strains. *bla*_{NDM} and *bla*_{KPC} genes were not found in any of the bacterial isolates. *bla*_{VIM} and *bla*_{OXA-48-like} gene distribution was significantly different (*p* < 0.05) among *K. pneumoniae* and *E. coli* isolates (Table 3).

Discussion

In the present study, the prevalence of ESBL-, AmpC-, and carbapenemase-associated genes were investigated by phenotypic and genotypic methods in clinical isolates of *K. pneumoniae* and *E. coli*. According to the phenotypic results, the prevalence of ESBL-producing *K. pneumoniae* and *E. coli* was notable. The genotypic method confirmed the phenotypic method results, except for 1 *E. coli* strain. Although this isolate harbored 1 ESBL-related gene, it did not show phenotypic resistance. Similarly, unexpressed ESBL genes in antibiotic-susceptible isolates were previously reported [18]. Horizontal gene transfer to other bacteria is possible via such strains [18].

Notable frequencies of ESBL-producing *K. pneumoniae* and *E. coli* strains were reported in Iran [19, 20]. It seems that the prevalence of ESBL-producing gram-negative bacteria is increasing in the country [20].

In the present study, 8 *K. pneumoniae* isolates carried 2 ESBL genes, and 3 isolates harbored 3 ESBL genes simultaneously. Moreover, the simultaneous presence of 2 and 3 ESBL genes was observed in 6 and 3 strains of *E. coli*, respectively. The copresence of ESBL genes among clinical *E. coli* isolates was previously stated [21].

In the present study, AmpC screening and confirmatory phenotypic tests produced different results. Similar to a previous study, a high rate of false-negative results was reported by phenotypic detection methods for AmpC [13]. In our study, AmpC-producing *K. pneumoniae* strains were higher than in a previous report from Iran [22]. However, a notable frequency of AmpC-producing *K. pneumoniae* isolates (43%) was reported among burnt patients [13]. Dissemination of AmpC β -lactamases between near half of clinical gram-negative bacilli has been reported [13, 23, 24]. In the present study, 6 AmpC-producing *K. pneumoniae* isolates carried *bla*_{DHA} and *bla*_{EBC} simultaneously, and copresence of *bla*_{DHA}, *bla*_{EBC}, and *bla*_{CIT} was observed in 2 isolates. Recently, the coexistence of AmpC β -lactamase genes in *K. pneumoniae* was reported for the first time [13]. To eradicate AmpC-producing bacteria, carbapenems can be used as primary agents, but AmpC β -lactamases might increase the MIC values of carbapenems and confer resistance to the carbapenem family [13, 25].

A frequent gene among carbapenemase-producing *K. pneumoniae* was *bla*_{VIM}, but only 1 harbored the *bla*_{IMP} gene. Similarly, in a previous study, the *bla*_{VIM} gene was more frequent than the *bla*_{IMP} gene [26]. The *bla*_{KPC} and *bla*_{NDM} genes were not detected in any of the investigated

strains, while the presence of *bla*_{KPC} was notable in *K. pneumoniae* isolates in Iran [27]. Also, in a study by Hosseinzadeh et al. [28], >10% of the isolates carried the *bla*_{NDM-1} gene.

Among carbapenemase-producing *K. pneumoniae*, 8 (23.5%) isolates simultaneously harbored *bla*_{VIM} and *bla*_{OXA-48-like} genes, and the copresence of *bla*_{VIM}, *bla*_{OXA-48-like}, and *bla*_{IMP} genes was detected in only 1 isolate (2.9%). Among carbapenemase-producing *E. coli* isolates, 8 (61.5%) carried *bla*_{VIM} and *bla*_{OXA-48-like} genes simultaneously. The *bla*_{OXA-48-like} gene was detected among all of them.

Our results indicated that some resistance genes were present amongst the resistant strains, which was statistically significant ($p < 0.05$). These appear to be the most frequent ESBL-, AmpC-, and carbapenemase-related genes in our region (Tables 1–3). Coexistence of ESBL, AmpC, and metallo- β -lactamases was reported amongst *A. baumannii* strains in 2013 [29]. Pokhrel et al. [30] showed the copresence of ESBLs and carbapenemase in clinical *E. coli* isolates in Nepal.

The current study shows that 2 *K. pneumoniae* isolates simultaneously carried 2 ESBL (*bla*_{CTX-M}, *bla*_{TEM} / *bla*_{CTX-M}, *bla*_{SHV}), 2 AmpC (*bla*_{DHA} and *bla*_{EBC}), and 2 carbapenemase genes (*bla*_{VIM} and *bla*_{OXA-48-like}). Moreover, coexistence of 3 ESBL (*bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV}), 1 AmpC (*bla*_{DHA}), and 3 carbapenemase genes (*bla*_{VIM}, *bla*_{OXA-48-like}, and *bla*_{IMP}) was seen in 1 of the studied isolates.

Conclusion

For the first time, we reported the occurrences of ESBL, AmpC, and carbapenemase genes in *K. pneumoniae* strains in Iran. In the present study, 2 isolates simultaneously carried 2 ESBL genes, 2 AmpC, and 2 carbapenemase genes. Moreover, coexistence of 3 ESBL, 1 AmpC, and 3 carbapenemase genes was observed in 1 *K. pneumoniae* isolate. Therefore, *K. pneumoniae* can be considered as a major pathogen and important hazard for public health. Up-to-date treatment with combination therapy and/or new antimicrobial agents, for example, might be helpful against such drug-resistant organisms.

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Statement of Ethics

This study was approved by the Ethics Committee of Ilam University of Medical Sciences.

Disclosure Statement

The authors declare that there is no conflict of interest.

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