Clin. Lab. 2023;69:XXX-XXX

ORIGINAL ARTICLE

The Prevalence of Virulence Factors among ESBLs-Producing *Klebsiella pneumoniae* Isolated from Khorramabad Hospitals, Iran

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SUMMARY

Background: ***Klebsiella pneumoniae*** **is one of the opportunistic bacteria that cause hospital infections. Various virulence factors are involved in its pathogenesis. The purpose of our study was to investigate the prevalence of virulence factor genes *entB, mrkD, magA, kfu, iutA, rmpA, ybtS,* and *k2* among ESBLs-producing *Klebsiella pneumoniae* isolated from clinical samples of Khorramabad hospitals in Iran.**
Methods: **In this descriptive, analytical study, the extended-spectrum β-lactamases (ESBLs) screening phenotypic test was performed by disc diffusion method, and virulence factor genes were detected by PCR method for *Klebsiella pneumoniae* isolates. Finally, the obtained data were statistically analyzed by SPSS software (version 21) using the chi-squared test.**
Results: **One hundred and ten *K. pneumoniae* strains were isolated from urine 69 (62.7%), sputum 16 (14.5%), blood 10 (9.1%), wound 9 (8.2%), tissue 3 (2.7%), body fluids 2 (1.8%), and catheter 1(0.9%). Based on an ESBL screening phenotypic test, 57 (51.8%) *K. pneumoniae* isolates were ESBLs-producing. Among 57 ESBLs-positive *K. pneumoniae*, the occurrence of *mrkD, entB, ybtS, iutA, kfu, k2*, and *rmpA* genes were 88 (80%), 87 (79.1%), 65 (59.1%), 42 (38.2%), 19 (17.3%), 5 (4.5%), 3 (2.7%), and 2 (1.8%), respectively.**
Conclusions: **The findings of this study revealed, there is no significant association between the occurrence of virulence genes or source of samples with production of ESBLs among *K. pneumoniae* isolates.**
(Clin. Lab. 2023;69:xx-xx. DOI: 10.7754/Clin.Lab.2023.230322)

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KEYWORDS

extended-spectrum β-lactamases (ESBLs), *Klebsiella pneumoniae*, virulence factors

INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) is a Gram-negative, non-motile, lactose-fermenting bacillus that has different antigens, including O and K [1,2]. Also, *K. pneumoniae* is one of the 5 most common Gram-negative bacteria in hospital infections. This organism was responsible for 7 to 10% of all hospital septicemias between 1997 and 2002 [3]. *K. pneumoniae* produces a number of virulence factors that are involved in its pathogenesis including adhesins, siderophores, antigen O, capsule, etc. Capsule is antigenically divided into 77 serotypes, which is the most important factor in the patho-

Manuscript accepted May 14, 2023

genesis of this bacterium [4-6]. This polysaccharide layer covers the surface of the bacterium as a dense mass and prevents the phagocytosis of the bacterium by phagocytes. The capsular antigens K1 and K2 present in the mouse peritoneal model possess significant virulence [7,8]. Adhesins are considered as additional virulence factors, like pili which have a protein structure and recognize a wide range of molecular motifs and cause bacteria to attach to mucosa surfaces [9]. *K. pneumoniae* produces two main types of pili. Pili type III is one of the most important adhesins on the surface of *K. pneumoniae*, which is composed of the main subunit protein MrkA and adhesin MrkD [10]. The *MrkD* gene is not conserved among *Klebsiella spp*, while the *MrkA* gene is conserved [11]. These pili bind to the side surfaces of epithelial cells of the trachea, human lung tissue, basement membrane components, and matrix proteins. This pilus causes agglutination of erythrocytes treated with tannic acid *in vitro*, and this type of hemagglutination can occur in the presence or absence of D-mannose [12,13]. This hemagglutination is called mannose-resistant hemagglutination. It is specifically attached to iron and subsequently absorbs it through receptors in the cell membrane. The results of previous studies have showed that *K. pneumoniae* is able to use more than one method to obtain iron [14]. Recently, the presence of an iron absorption system by yersiniabactin of *K. pneumoniae* has been described [15]. The virulence factors are encoded by *ybts* (encoding yersiniabactin), *entB* (encoding enterobactin), *mrkD* (encoding pili adhesin), *k2* (encoding capsule type 2), *magA* (encoding capsule type 1), *iutA* (encoding siderophore hydroxymate), *kfu* (encoding ferric iron absorption), *rmpA* (regulates mucoid phenotype), and *allS* (allantoin metabolism) [7,15-17]. Cephalosporins are the first line treatment of *Klebsiella* infections [18]. The occurrence of bacterial resistance is very widespread not only in *K. pneumoniae*, but also in all bacteria today. Specially, with the emergence of *K. pneumoniae* which are able to hydrolyze cephalosporins and even carbapenems, the scientific community should be alerted about treatment choices [19,20]. The increase of antibiotic resistance rate among common pathogenic bacteria is a serious threat to the control of infectious diseases. Bacterial resistance to antibiotics and the global spread of resistant strains is one of the main problems affecting the health care systems. Among antibiotics, beta-lactams are the most abundant and diverse antibiotics used [21]. One of the most important mechanisms of resistance in Gram-negative bacteria is the production of extended-spectrum β-lactamases (ESBLs), which are capable of hydrolyzing broad-spectrum cephalosporins such as cefotaxime, ceftriaxone, and ceftazidime. These enzymes are often encoded and transferred by plasmids [22]. These enzymes have been recognized in a variety of Gram-negative bacilli. Gram-negative bacteria resistant to extended-spectrum beta-lactam antibiotics have rapidly spread in the last two decades, which is the result of the spread of ESBLs in the Enterobacteriaceae family. Since the genes encoding these enzymes are easily transferred between the members of Enterobacteriaceae family through plasmids, they can facilitate the development of resistance not only to beta-lactams, but also to other antibiotics such as quinolones and aminoglycosides [23-25]. The aim of the current study was to determine the occurrence of virulence factor genes *entB, mrkD, magA, kfu, iutA, rmpA, ybtS,* and *k2* among ESBLs-producing *K. pneumoniae* isolated from clinical samples of Khorramabad hospitals in Iran.

MATERIALS AND METHODS

Sample collection

In this descriptive-analytical study, *K. pneumoniae* was collected from the clinical samples of hospitals in Khorramabad city (western of Iran) in 2018. After culturing the samples on blood agar and MacConkey agar plates (Merck, Germany), the cultured colonies were identified through biochemical and microbial standard tests [26].

Extended-spectrum β-lactamases (ESBLs) screening phenotypic test

Detection of ESBLs-producing isolates was performed by disc diffusion method using cefotaxime, ceftazidime, ceftriaxone, cefpodoxime and aztreonam, and cefotaxime + clavulanic acid, ceftazidime + clavulanic acid, cefpodoxime + clavulanic acid discs (MAST, UK) on Mueller-Hinton agar (Merck, Germany). After incubating plates for 18 to 24 hours at 37℃, if the difference in the diameter of the non-growth zone around each combined disc (with clavulanic acid) compared to corresponding disc (without clavulanic acid) was at least 5 mm, it would indicate the presence of extended-spectrum β-lactamase enzymes [27]. *K. pneumoniae* ATCC 600703 and *Escherichia. coli* ATCC 25922 were used as positive and negative control strains, respectively.

Detection of virulence genes

After extracting bacterial DNA using the SinnaPure DNA extraction kit (Sina Clone, Iran) according to the instructions in the kit, the detection of *entB, mrkD, magA, kfu, iutA, rmpA,* and *K2* genes was performed by the PCR technique. The sequence of the primers [7] used and the temperature program for the amplification of the genes studied are shown in Tables 1 and 2, respectively. The PCR products were electrophoresed on 1% agarose gel and then photographed using a gel doc device (BioDocAnalyse, Biometra).

Statistical analysis

Data were statistically analyzed using SPSS software (version 21) and chi-squared test. p-values less than or equal to 0.05 was considered as significant.

Table 1. Primers used to detect virulence factor genes.

Primer	DNA sequence (5' to 3')	Amplicon size (bp)	Target gene product/function
***mrkD*-F**	**AAGCTATCGCTGTACTTCCGGCA**	**340**	**Fimbriae**
***mrkD*-R**	**GGCGTTGGCGCTCAGATAGG**		
***entB*-F**	**GTCAACTGGGCCTTTGAGCCGTC**	**400**	**Siderophore**
***entB*-R**	**TATGGGCGTAAACGCCGGTGAT**		
rmpA-F	**CATAAGAGTATTGGTTGACAG**	**461**	**Regulator of mucoid phenotype A**
rmpA-R	**CTTGCATGAGCCATCTTTCA**		
***k2*-F**	**CAACCATGGTGGTCGATTAG**	**531**	**Capsular serotype K2 and hypermucoviscosity phenotype**
***k2*-R**	**TGGTAGCCATATCCCTTTGG**		
***kfu*-F**	**GGCCTTTGTCCAGAGCTACG**	**638**	**Iron transport and phosphotransferase function**
***kfu*-R**	**GGGTCTGGCGCAGAGTATGC**		
***ybtS*-F**	**GACGGAAACAGCACGGTAAA**	**242**	**Yersiniabacten**
***ybtS*-R**	**GAGCATAATAAGGCGAAAGA**		
***iutA*-F**	**GGGAAAGGCTTCTCTGCCAT**	**920**	**Siderophore**
***iutA*-R**	**TTATTCGCCACCACGCTCTT**		
***magA*-F**	**GGTGCTCTTTACATCATTGC**	**283**	**Capsular serotype K1 and hypermucoviscosity phenotype**
***magA*-R**	**GCAATGGCCATTTGCGTTAG**		

Table 2. Temperature program used to examine virulence factor genes.

Genes	Thermocycler program
Multiplex PCR *rmpA, iutA, k2* genes	**initial denaturation at 95°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, 58°C for 90 seconds, and 72°C for 60 seconds, and elongation at 72°C for 10 minutes**
Multiplex PCR *entB, mrkD* and *kfu* genes	**initial denaturation at 95°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, 60°C for 90 seconds, and 72°C for 60 seconds, and elongation at 72°C for 10 minutes**
magA	**initial denaturation at 95°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, 56°C for 90 seconds, and 72°C for 60 seconds, and elongation at 72°C for 10 minutes**

Table 3. The frequency of the virulence genes among ESBLs-producing *Klebsiella pneumoniae.*

	Total no. of ESBLs-positive isolates = 57			
			n (%)	p-value
Virulence genes	***K2***	+	3 (5.3)	0.70
		-	54 (94.7)	
	magA	+	2 (3.5)	0.17
		-	55 (96.5)	
	iutA	+	24 (42.1)	0.38
		-	33 (57.9)	
	kfu	+	11 (19.3)	0.56
		-	46 (80.7)	
	rmpA	+	2 (3.5)	0.60
		-	55 (96.5)	
	mrkD	+	44 (77.2)	0.44
		-	13 (22.8)	
	entB	+	44 (77.2)	0.61
		-	13 (22.8)	
	ybtS	+	36 (63.2)	0.37
		-	21 (36.8)	

Table 4. Relationship between the type of samples and the production of ESBLs in isolates carrying virulence genes.

Genes	*ybtS* n = 65 (%)			*iutA* n = 42 (%)			*kfu* n = 19 (%)			*k2* n = 5 (%)			*rmp A* n = 3 (%)		
Samples	**ESBL+ n = 36**	**ESBL- n = 29**	**p-value**	**ESBL+ n = 24**	**ESBL- n = 18**	**p-value**	**ESBL+ n = 11**	**ESBL- n = 8**	**p-value**	**ESBL+ n = 3**	**ESBL- n = 2**	**p-value**	**ESBL+ n = 2**	**ESBL- n = 1**	**p-value**
Urine n = 69	24 (66.7)	14 (48.3)	0.167	17 (70.8)	7 (38.9)	0.131	5 (45.5)	4 (50)	0.146	3 (100)	1 (50)	0.171	2 (100)	0 (0)	0.083
Sputum n = 16	7 (19.4)	6 (20.7)		3 (12.5)	6 (33.3)		4 (36.4)	0 (0)		-	-		-	-	
Blood n = 10	3 (8.3)	2 (6.9)		3 (12.5)	2 (11.1)		1 (9.1)	0 (0)		0 (0)	1 (50)		0 (0)	1 (100)	
Wound n = 9	1 (2.8)	6 (20.7)		1 (4.2)	3 (16.7)		1 (9.1)	3 (37.5)		-	-		-	-	
Tissue n = 3	1 (2.8)	0 (0)		-	-		0 (0)	1 (12.5)		-	-		-	-	
Body fluids n = 2	0 (0)	1 (3.4)		-	-		-	-		-	-		-	-	
Catheter n = 1	-	-		-	-		-	-		-	-		-	-	

Table 4. Relationship between the type of samples and the production of ESBLs in isolates carrying virulence genes (continued).

Genes	Samples	*entB* n = 87 (%)			*mrkD* n = 88 (%)		
		ESBL+ n = 44	ESBL- n = 43	p-value	ESBL+ n = 44	ESBL- n = 44	p-value
				0.236			0.245
	Urine n = 69	29 (65.9)	25 (58.1)		29 (65.9)	26 (59.1)	
	Sputum n = 16	8 (18.2)	5 (11.6)		8 (18.2)	5 (11.4)	
	Blood n = 10	4 (9.1)	3 (7)		4 (9.1)	3 (6.8)	
	Wound n = 9	1 (2.3)	6 (14)		1 (2.3)	6 (13.6)	
	Tissue n = 3	2 (4.5)	1 (2.3)		2 (4.5)	1 (2.3)	
	Body fluids n = 2	0 (0)	2 (4.7)		0 (0)	2 (4.5)	
	Catheter n = 1	0 (0)	1 (2.3)		0 (0)	1 (2.3)	

RESULTS

One hundred and ten *K. pneumoniae* strains were isolated from urine 69 (62.7%), sputum 16 (14.5%), blood 10 (9.1%), wound 9 (8.2%), tissue 3 (2.7%), body fluids 2 (1.8%), and catheter 1 (0.9%). Based on the ESBL screening phenotypic test, 57 (51.8%) *K. pneumoniae* isolates were ESBL positive. PCR results of 110 *K. pneumoniae* showed, the occurrence of *mrkD*, *entB*, *ybtS*, *iutA*, *kfu*, *k2*, *rmpA*, and *magA* genes were 88 (80%), 87 (79.1%), 65 (59.1%), 42 (38.2%), 19 (17.3%), 5 (4.5%), 3 (2.7%), and 2 (1.8%), respectively. Table 3 shows total number of virulence genes and the ESBLs-production status. In this context, there is no considerable relationship between the presence of the virulence genes and ESBLs production (p-value > 0.05). As shown in Table 4, the results of the statistical analysis demonstrated, there is no significant association between the source of samples and ESBLs-positive phenotype of isolates (p-value > 0.05).

DISCUSSION

In the last few decades, due to the irregular clinical use of antibiotics worldwide, hospital-acquired *K. pneumoniae* isolates producing ESBLs have spread more and more, making the treatment of infectious diseases challenging [28]. The increasing rate of ESBLs-producing isolates is one of the most important reasons to be concerned about isolates of *K. pneumoniae* and other members of the Enterobacteriaceae family [29,30]. In the present study, the frequency of ESBLs-producing *K. pneumoniae* isolates was 51.8%. In other studies, conducted by Muller-Schulte et al. in Bouake, central Cote d'Ivoire [31], and Vaziri et al. in Kermanshah, Iran [32], the prevalence of ESBLs-producing isolates was 84% and 41.3%, respectively. This discrepancy could be partly explained by the difference in the pattern of antibiotic usage in hospitals, the transfer of various genes causing resistance on mobile genetic elements such as plasmids and bacteriophages, and also, the dissimilar occurrence of resistance genes between Gram-negative bacilli in different regions [33]. Our PCR results revealed that the *entB* and *mrkD* genes had the highest frequency among the tested genes encoding virulence factors in ESBLs-positive isolates (Table 3). The results of our study were consistent with those of the study by

Soltani et al., in which the *entB* gene (95.1%) was the most prevalent. ESBLs-positive isolates showed a significant association with the *mrkD* and *fimH* and hypermucoviscosity *rmpA* genes [34]. Ahmed et al. concluded that the *entB* gene was commonly found in all ESBLs-producing *K. pneumoniae* [35], Ahmadi et al. reported the most commonly detected virulence gene (94%) among ESBL-positive *K. pneumoniae* was the *mrkD* [36]. In addition, the results of that study reveal that the most prevalent virulence genes were observed in clinical samples isolated from burns and blood infections that were resistant to the third-generation of cephalosporins, which indicates a positive connection between antibiotic resistance to cephalosporins and the occurrence of virulence factors [35]. In spite of our findings, Osman et al. report that the most prevalent virulence genes were *magA*, *rmpA*, *kfu*, and *uge* [37]. Data obtained from studies performed by Padilla et al. [38] and Gharrah et al. [39] demonstrated that there is a significant association between ESBL production and virulence factors among *K. pneumoniae* isolates. The results of statistical analysis for our study demonstrated, there was no significant connection between the occurrence of virulence genes tested or the source of samples (urine, blood,…) with ESBL-positive phenotypes of isolates (Table 3 and 4). When interpreting, it should be remembered that many ESBLs which hydrolyze broad-spectrum cephalosporins are carried on mobile genetic elements such as conjugable plasmids. Therefore, the frequency of these enzymes naturally varies from region to region. This subject is partly dependent on the selective pressures resulting from antibiotic use and distribution of resistant clones in hospital settings. However, due to the crucial role of some virulence genes in maintaining the survival and life of bacteria, their nature are chromosome-encoded, which affects their frequency. It hypothesizes that their presence and transferring between isolates should be non-aligned with virulence factors. In addition, 69/110 (62.7%) of the specimens tested were urine. It is clear, due to the imbalance between the number of sample types (urine, sputum, blood, …), no statistically significant difference could be found in terms of prevalence of virulence genes between the sample types. To our knowledge, the present study is the first report which aimed virulence factors of *K. pneumoniae* in western of Iran. However, limitation on sample size and non investigation of other members of Enterobacteriaceae family were the weakness of the study. The results of this study and previous studies show that the occurrence of virulence factors depend on the geographical area. In addition, the extent of antibiotic resistances can be variable. Therefore, our insight regarding the major factors involved in the pathogenesis of common bacteria and their resistance profiles could be useful to determine the strategy of health care systems to combat and eradicate community/hospital-acquired infections. We suggest that further and complementary studies be conducted to examine the association between the presence of *Klebsiella* virulence factor genes and resistance to other antibiotic families.

CONCLUSION

In conclusion, the findings of this study revealed, there is no significant association between the tested virulence genes or source of samples with production of ESBLs among *K. pneumoniae* isolates.

Acknowledgment/Source of Funds:
This work was supported by the Lorestan University of Medical Sciences, Khorramabad, Iran.

Availability of Data and Materials:
All data generated or analyzed during this study are included in this published article.

Consent for Publication:
Not applicable.

Ethical Statement:
This research was agreed upon by the Ethics Committee of the Lorestan University of Medical Sciences, Iran (IR.LUMS.REC.1395.161).

Declaration of Interest:
The authors declare that they have no competing interests.

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