Jundishapur J Microbiol. 2018 April; 11(4):e61285. doi: 10.5812/jjm.61285.
Published online 2018 April 20. **Research Article**

Comparative Prevalence of *bla*$_{\text{CTX-M-15}}$ Gene with Virulence Genes and Serotypes in *Klebsiella pneumoniae*

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Received 2017 October 23; **Revised** 2018 January 18; **Accepted** 2018 February 26.

Abstract

Background: A newly emerged hypervirulent strain of *Klebsiella pneumoniae* has caused great concern globally; however, its molecular characteristics have been rarely reported in Iran.
Objectives: The goal of this study was to detect the virulence determinants and serotypes of *K. pneumoniae* and to evaluate the association among selected virulence traits and *bla*$_{\text{CTX-M-15}}$ gene in southeastern Iran.
Methods: One hundred and three non-duplicate *K. pneumoniae* strains were isolated from clinical samples. The isolates were identified by standard bacteriological tests. Confirmed isolates were examined to detect a selection of virulence genes (*wabG*, *rmpA* and *iucB*) and serotypes (K1, K2, K5 and K20) by PCR. The isolates were studied foe the presence of beta-lactamase (*bla*$_{\text{CTX-M-15}}$) gene. SPSS software (version 19.0) was used for data analysis.
Results: Among the 103 *K. pneumoniae* isolates, 61 (59.2%) isolates were positive for *wabG*, 4 (3.9%) for *iucB* and 3 (2.9%) for *rmpA* genes. The presence of K20 in 3.9% (4/103) of the isolates represented the most prevalent. Only 3 (2.9%) isolates possessed the K1 serotype, while K2 and K5 serotypes were not detected in any isolate. The *bla*$_{\text{CTX-M-15}}$ gene was detected in 47 (45.6%) isolates. *bla*$_{\text{CTX-M-15}}$-positive isolates showed a higher prevalence of *wabG* among the studied isolates ($P < 0.05$).
Conclusions: Our data indicate a correlation between presence of virulence gene and *bla*$_{\text{CTX-M-15}}$ in *K. pneumonia* isolates. Further research should be undertaken to unravel aspects of both virulence factors and antibiotic resistance which may probably contribute to managing future spread of infectious diseases.

Keywords: Antibiotic Resistance, Serogroup, Virulence Factors, Iran, *Klebsiella pneumoniae*

1. Background

Classic *Klebsiella pneumoniae* are multidrug-resistant strains associated with nosocomial infections outbreak and its hypervirulent strains are related to severe community-acquired infections (1, 2). During the past decade, CTX-M enzymes have become the most prevalent extended spectrum beta-lactamase (ESBL) enzymes in nosocomial infections caused by *K. pneumoniae* (3, 4). *Klebsiella pneumoniae* strains with plasmids harbouring *bla*$_{\text{CTX-M-15}}$ have been detected in clinical isolates worldwide (5).

Bacteria can acquire antimicrobial resistance by the spread of transmissible plasmids, which may also carry virulent traits. The acquisition of antibiotic resistance and virulence determinants may represent a survival benefit to the bacteria (6). Several virulence genes such as the regulator of mucoid phenotype A gene (*rmpA*), iron acquisition system aerobactin (*iucB*) and lipopolysaccharide synthesis (*wabG*) have been detected and determined to be associated with the hypervirulent variant of *K. pneumoniae*. Both aerobactin system and regulator of mucoid phenotype A gene are found almost in all reported hypervirulent variants of *K. pneumoniae* strains located on a large plasmid. The important roles of *rmpA* and *wabG* genes are in capsular polysaccharide and lipopolysaccharide syntheses, respectively (7, 8).

Capsular polysaccharide (K antigen) is a major virulence factor responsible phagocytosis and serum killing resistance. At least 78 capsular serotypes have been identified while, serotype-related variation in infection severity have been observed. Strains with capsular serotypes K1 and, to a lesser extent, K2, K5, K20 and K54, have been recognized as the predominant virulent strains (8, 9).

2. Objectives

The aim of this study was to identify the mucoviscosity, iron acquisition, lipopolysaccharide synthesis and serotypes of *K. pneumoniae* strains isolated from clinical specimens and to evaluate the association among virulence traits and $bla_{\text{CTX-M-15}}$ gene.

3. Methods

3.1. Ethics Statement

This study has been approved by the ethics committee of Kerman University of Medical Sciences (Code Number: IR.KMU.REC.1394.496), and written consent has been obtained from all patients.

3.2. Bacteria and DNA Extract

From October 2014 to May 2015, 103 non-duplicate non-consecutive *K. pneumoniae* were collected from patients referred to different clinical laboratories and hospitals in Kerman city (southeastern Iran). Clinical isolates were mostly from urine, wound, tracheal secretions and blood. The isolates were identified by standard biochemical and bacteriological methods. Isolates were then preserved at -70°C in brain-heart infusion (BHI) (HiMedia, India) broth with 30% glycerol until further tests. Bacterial genomic DNA was extracted from isolates using a kit (Topaz gene research, Iran) according to the manufacturer's instructions.

3.3. Virulence Genes

Specific PCR primers for the detection of *rmpA*, *wabG* and *iucB* genes were previously described by Chen et al. (8).

3.4. Serotypes

K1, K2, K5 and K20 serotypes were identified by the detection of capsular polysaccharide (K serotype-specific) *wzx* and *wzy* alleles, using the multiplex PCR method as previously described (8).

3.5. Detection of $bla_{\text{CTX-M-15}}$ Gene

All isolates were screened by PCR for the $bla_{\text{CTX-M-15}}$ gene, as described by Messai et al. (10).

3.6. String Test

The presence of hypermucoviscosity was evaluated using the string test, and a positive result was defined as formation of a viscous capsular string > 5 mm in length by stretching bacterial colonies on an agar plate (11).

3.7. Statistical Analysis

Descriptive and inferential statics were used for data analyse. Differences in virulence genes and serotypes with antimicrobial resistance gene were compared using, SPSS version 19.0 (SPSS, Inc), with a P value of $P < 0.05$ indicating statistical significance.

4. Results

The *K. pneumoniae* isolates were screened for the presence of virulence genes. In total, 62 out of 103 (60.2%) *K. pneumoniae* isolates harbored at least one of the virulence genes chosen for analysis. The most prevalent virulence gene was *wabG* (59.2%; 61/103), followed by *iucB* (3.9%; 4/103). The regulator of mucoid phenotype encoding gene *rmpA* was present in 2.9% (3/103) of the isolates (Figures 1, 2) (Table 1).

Table 1. Number and Percent of Serotypes, Virulence and $bla_{\text{CTX-M-15}}$ Genes Present in *K. pneumoniae* Isolates

Variables	Gene	Total, No. (%)
Serotypes	K1	3 (2.9)
	K2	-
	K5	-
	K20	4 (3.9)
Virulence trait	*wabG*	61 (59.2)
	iucB	4 (3.9)
	rmpA	3 (2.9)
ESBL	$bla_{\text{CTX-M-15}}$	47 (45.6)

Overall, 7 of 103 (6.8%) *K. pneumoniae* isolates were positive for at least one of the examined serotype genes. The presence of K20 in 3.9% (4/103) of the isolates was the most prevalent represented. The K1 serotype was the least prevalent in three isolates (Figure 3). None of the *K. pneumoniae* isolates were positive for K2 and K5 serotypes. Among the isolates positive for K1 serotype, 0.9% (1/103) was in combination with *wabG*, *iucB* and *rmpA* genes (hypervirulent), 0.9% (1/103) of isolates in combination with $bla_{\text{CTX-M-15}}$, *wabG*, *iucB* and *rmpA* genes and 0.9% (1/103) was accompanied by $bla_{\text{CTX-M-15}}$, *wabG* and *iucB* genes. Also, among the isolates that possessed the K20 serotype, 2.9% (3/103) were in combination with *wabG* gene.

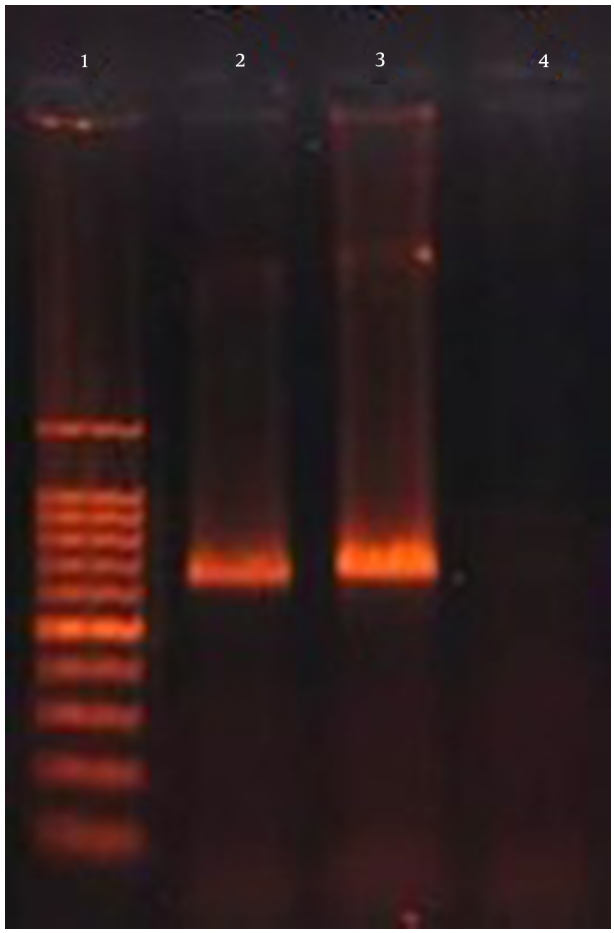

Figure 1. Lane 1, 100 bp DNA Marker; Lane 2, 3, an Amplification of *wabG* Gene (683 bp); Lane 4, Negative Control

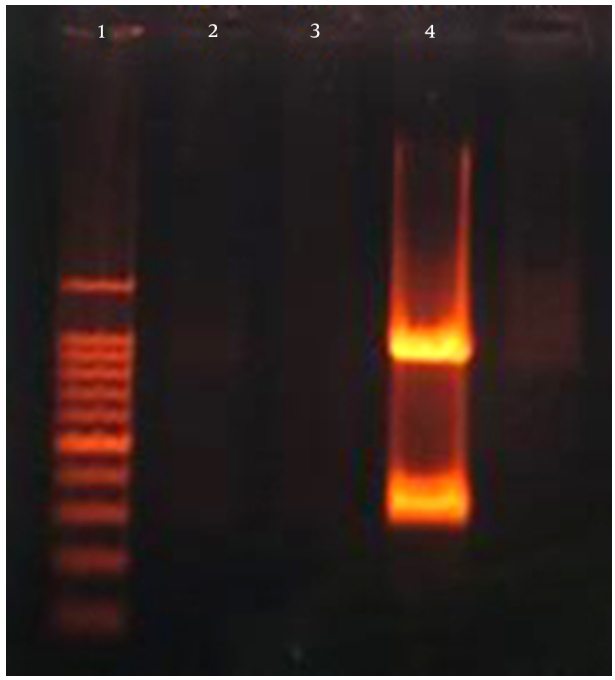

Figure 2. Lane 1, 100 bp DNA Marker; Lane 2, Negative Control; Lane 4, Amplification of *rmpA* Gene (322 bp) and *iucB* Gene (948 bp)

Table 2. Prevalence of Virulence Genes and Serotypes According to Isolates Possessed $bla_{CTX\text{-}M\text{-}15}$ Gene in *Klebsiella pneumoniae* Isolates

Variables	Virulence Genes and Serotypes Pattern	No.
$bla_{CTX\text{-}M\text{-}15}$ [a]	*wabG*, *rmpA*, *iucB*, K1	1
	wabG, *iucB*, K1	1
	wabG, *rmpA*, *iucB*	1
	wabG, K20	2
	wabG	23
	-	19
Total		47

[a] Isolates possessed $bla_{CTX\text{-}M\text{-}15}$ gene.

Based on the results of the string test, hypervirulent phenotypes were identified in 3 (2.9%) of the isolates, whit only one (0.9%) isolate having the characteristic hypervirulent phenotype, was positive for K1, *rmpA*, *wabG* and *iucB* genes. The ESBL encoding gene $bla_{CTX\text{-}M\text{-}15}$ was observed in 45.6% (47/103) of isolates (Figure 4 and Table 1). Of the 47 *K. pneumoniae* isolates that possessed $bla_{CTX\text{-}M\text{-}15}$ gene, 28 (59.6%) isolates were positive for *wabG* gene. Bivariate analysis confirmed that this difference between the number of isolates was significant (P = 0.000). Among 47 isolates that possessed $bla_{CTX\text{-}M\text{-}15}$ gene, three isolates were positive for *rmpA* gene (P > 0.05). There was no significant difference in $bla_{CTX\text{-}M\text{-}15}$ gene to *iucB* gene (3/47 isolates) (P > 0.05). In addition, there was no association between the presence of $bla_{CTX\text{-}M\text{-}15}$ gene and the K1 (2/47 isolates) and K20 (2/47 isolates) serotypes (P > 0.05).

5. Discussion

Klebsiella pneumoniae is known as an important reservoir for various types of ESBL enzymes, and has spread worldwide (12). The high rate of CTX-M producing *K. pneumoniae* prevalence and its widespread dissemination in Iran is causing concern (13). Virulence genes thought to be associated with invasive community-acquired infections include lipopolysaccharide synthesis, iron acquisition system, hypermucoidy and specific polysaccharide capsule serotypes (2). The present study showed a correlation between the epidemiology of specific virulence genetic traits

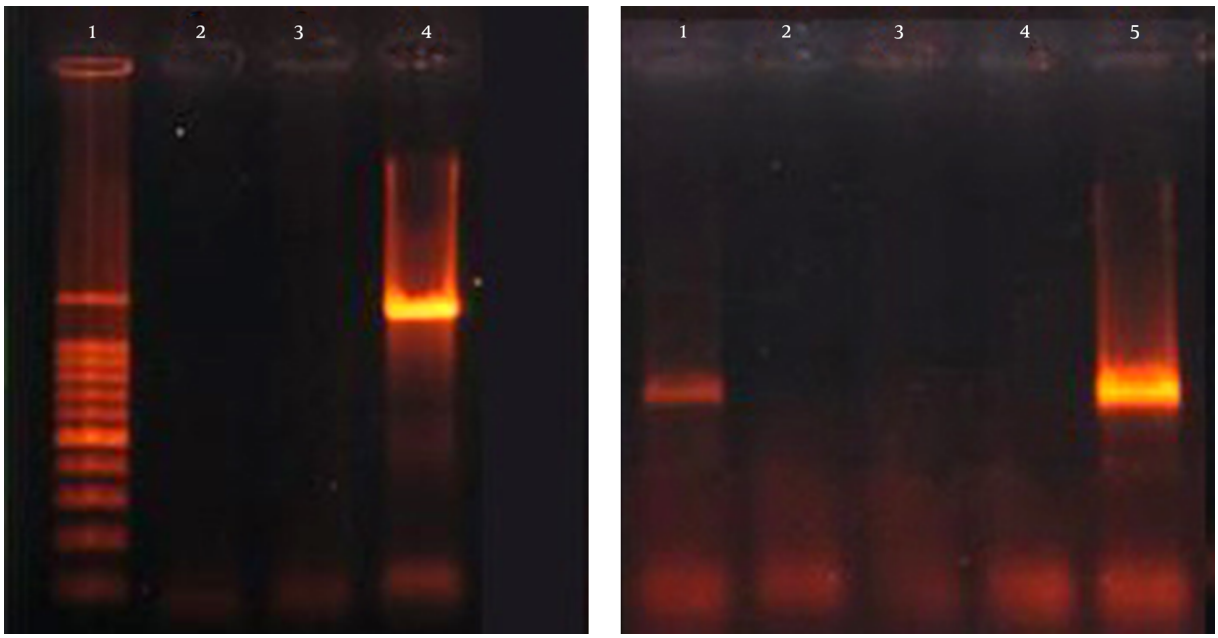

Figure 3. A, Lane 1, 100 bp DNA Marker; Lane 2, Negative Control; Lane 4, Amplification of K1 (1283 bp); B, Lane 1, 5, Amplification of K20 (741 bp); Lane 2, Negative Control

and serotypes with $bla_{\text{CTX-M-15}}$ gene; a first step toward understanding whether there is a link between virulence and resistance. The results from previous researches have shown that there is a link between resistance and virulence in *Escherichia coli* isolates (6). Lavigne et al. demonstrated that 14 virulence factors were less prevalent in ESBL isolates than in susceptible *E. coli* isolates (14). In another study by Lee et al. showed that CTX-M producers of E. coli isolates had fewer virulence factors than TEM-producing isolates (15).

The results showed a high (45.6%) prevalence of $bla_{\text{CTX-M-15}}$ gene among the *K. pneumoniae* isolates. The existence of lipopolysaccharide synthesis encoding gene (*wabG* gene) was one of the most common virulence genes identified among the studied isolates. The findings by Chen et al. showed that there was high rate of *wabG* gene among 327 *K. pneumoniae* clinical isolates in China (8). According to the results of the current study, K1 serotype was detected in ~3% of the isolates. Lipopolysaccharide and capsular polysaccharide components are important pathogenic traits in K. pneumonia-caused bacteremia and pneumonia (16). The K1 strains were *wabG*, *iucB*, *rmpA* and $bla_{\text{CTX-M-15}}$-positive genes, which indicate that most of the K1 isolates tested in this study, were closely related to hyper-virulence and ESBL. Previous studies have shown that the K1 serotype *K. pneumoniae* isolated from liver abscesses is less resistant to antibiotics when compared with other serotypes (17, 18), whereas previously published data indicated that the K1 serotype is associated with antibiotic resistance (19, 20).

Of the virulence genes, *wabG* was significantly more common in strains producing the $bla_{\text{CTX-M-15}}$ group ESBLs. Derakhshan et al. compared the occurrence of *rmpA* and *wcaG* genes with the production of class 1 integron (*intl1*) among *K. pneumoniae* strains isolated from clinical specimens. The prevalence of *wcaG* was more frequent in the intl1 producing *K. pneumoniae* (21). The findings of this study indicated that the numbers of *wabG*-carrying isolates were more common in strains producing the $bla_{\text{CTX-M-15}}$ gene ($P < 0.05$). The results of the current study showed that the *rmpA* gene was detected in quite a low frequency compared to other isolates. The *rmpA* gene encodes a transcriptional activator of capsular polysaccharide which is one of the major contributing factors of virulence in *K. pneumoniae*, promoting biofilm formation and is closely associated with the hypermucoviscous phenotype (8, 22). Yu et al., showed that *rmpA*-positive isolates were related to the clinical syndrome caused by invasive strains and with the hypermucoviscous phenotype (23). Another study that demonstrated the relationship between *rmpA*, virulence and specific polysaccharide capsule serotypes is yet to be elucidated (9).

In the present study, the aerobactin encoding gene *iucB* was present in 3.9% of isolates. Several molecular epidemiologic studies were suggestive that hypervirulent *K. pneumonia* strains produced more aerobactin than classical *K.*

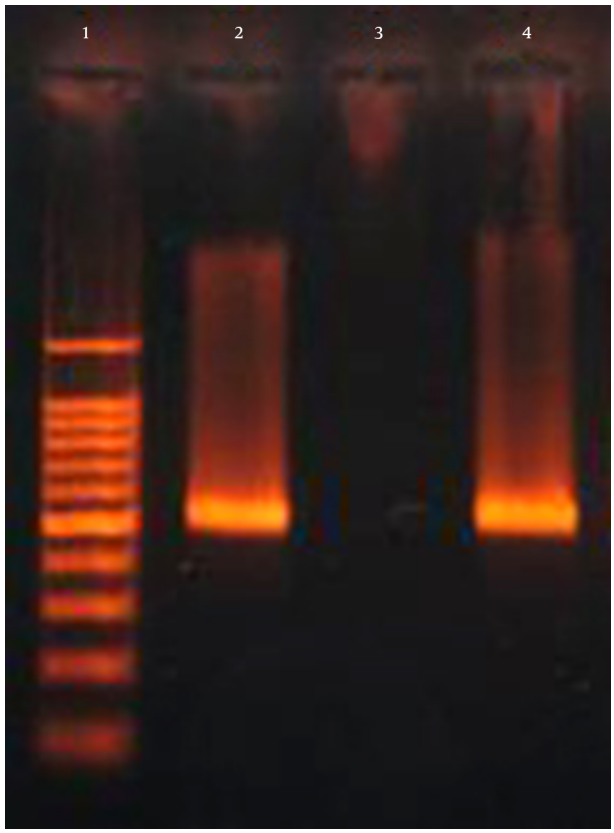

Figure 4. Lane 1, 100 bp DNA Marker; Lane 2, 4, Amplification of $bla_{CTX-M-15}$ Gene (550 bp); Lane 3, Negative Control

pneumoniae strains and it may be that this factor enhanced the virulence of hypervirulent *K. pneumoniae* (24, 25). In this regard, Li et al. suggested that hypervirulent *K. pneumoniae* has the potential to acquire significant antibiotic resistance (26).

6. Conclusions

In summary, the data presented in this study suggest that $bla_{CTX-M-15}$ producing *K. pneumoniae* contain high levels of *wabG* virulence gene. However, the association of virulence traits and antibiotic resistance in different studies are not clear. More in depth molecular epidemiologic researches on the genetics between virulence factors and antibiotic resistance are sorely needed, to fully understand the relationship between virulence and resistance genes in *K. pneumoniae* strains.

Acknowledgments

This work was supported by from research center for tropical and infectious diseases, Kerman University of Medical Sciences, Kerman, Iran (No: 94.658).

Footnotes

Authors' Contribution: Hesam Alizade and Mohammad Reza Aflatoonian, study concept and design, the development of the study; Hesam Alizade, Maziar Jajarmi and Saeed Shoja, performing experimental procedures; Hesam Alizade, Reza Ghanbarpour and Davood Kalantar-Neyestanaki, data interpretation, manuscript drafting and manuscript revision.

Conflict of Interest: The authors declare that they have no conflict of interest.

Role of the Sponsor: The funding organization had no role in the design and conduct of the study; collection, management, and analysis of data; and/or preparation, review, and approval of the manuscript. The research center for tropical and infectious diseases, Kerman University of Medical Sciences, Iran is acknowledged for funding assistance on this project.

Financial Disclosure: There was no financial disclosure to report.

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