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RESEARCH ARTICLE

Frequency of Iron Uptake Proteins Related Genes Among *Klebsiella pneumoniae* Isolates

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Abstract:

Aims:

The present study aimed to evaluate Iron uptake protein-related genes in clinical and environmental *Klebsiella pneumoniae* isolates.

Background:

Klebsiella pneumoniae as an opportunistic pathogen cause infections in immunocompromised patients. Iron uptake systems play an important role in the pathogenesis of *Klebsiella pneumoniae*.

Objectives:

This study was designed to investigate the prevalence of iron uptake coding genes among isolates of *Klebsiella pneumoniae*.

Materials and Methods:

A total of 300 isolates of *Klebsiella pneumoniae* including 150 clinical isolates and 150 environmental isolates were selected. Finally, the frequency of *iroN*, *iucD*, *kfuA*, *hmuR*, and *ybt* [*yHPI*] genes were detected by PCR method.

Results:

The frequency of *kfuA*, *iucD*, *iroN*, *yHPI* in clinical isolates were 33.3%, 16.7%, 24.7%, 15.3%, respectively and these genes among environmental isolates were 20.7%, 6%, 49.3% and 0.7%, respectively. Among the clinical isolates, the most frequency genes were *kfuA* gene [50 isolates] and after that *iroN* [37 isolates], *iucD* [25 isolates] and *yHPI* [23 isolates], the genes with the most frequency among environmental isolates were *iroN* gene [74 isolates] and following that *kfuA* [31 isolates], *iucD* [9 isolates] and *yHPI* [1 isolate]. No *hmuR* positive samples among all clinical or environmental isolates were found.

Conclusion:

The result of this study showed that because of the high frequency of ferric iron system coding gene *kfu* among clinical isolates, this system might play an important role in the survival of bacteria against its host.

Keywords: : *Klebsiella pneumoniae*, Iron uptake gene, *IroN*, *Iucd*, *Kfua*, *Hmur*, *Aybt*, PCR.

Article History

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1. INTRODUCTION

Klebsiella pneumoniae is a gram-negative bacillus belong-

ing to the Enterobacteriaceae family and, as an opportunistic pathogen can be found in water resources, soils and mucosal surfaces of mammals, as well as in the gastric, eye, respiratory and genital mucosa in humans [1, 2].

K. pneumoniae can be isolated from the upper respiratory

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tract and gastrointestinal tract in 5% of healthy people. This organism forms part of the normal microflora of the human body; about one-third of people are intestinal carriers of this microbe [2, 3]. *K. pneumoniae* is one of the causes of nosocomial infections; the colonization of this bacterium in hospitalized patients is more than outpatient reportedly. *K. pneumoniae* is an etiology of a wide range of infections including septicemia, pneumonia, Urinary Tract Infection (UTI), meningitis, and purulent abscesses in various organs, especially liver abscesses, Surgical Site Infection (SSI) and conjunctivitis [4]. This bacterium occasionally causes UTI and bacteremia along with localized damage in patients with immunodeficiency and catheter [5]. *K. pneumoniae* accounts for about 1% of bacterial pneumonia and can cause the production of necrotic dense and hemorrhagic centers in the lung [6, 7]. The most important virulence factors known in *K. pneumoniae* include polysaccharide capsules [77 serotypes], lipopolysaccharides (8 serotypes), adhesins and the iron uptake system [8]. The iron uptake system, behind the capsule, is the second most important virulence factor in *K. pneumoniae* [9]. The bacterial growth is inhibited in the host body not only by host defense mechanisms but also by available iron stores. Iron is an essential factor in bacterial growth, whose uptake is essential for the survival of pathogenic bacteria in the host body [10]. The iron is beneficial in the nutrition and growth of bacteria and is also a vital cofactor in defense enzymes against oxidative stresses, such as the components of superoxide dismutase, catalase, and peroxidase [11, 12]. Since this element is bonded to extracellular proteins such as hemoglobin, myoglobin, hemosiderin, ferritin and also proteins with high affinity for iron such as lactoferrin and transferrin, so the iron stores available for bacteria are limited, as well as free iron (10-18 mol) level is several thousand times less than the level required for bacterial growth. The bacterium absorbs iron, either directly or by secretion of molecules with high affinity, low molecular weight and iron-chelating agent, known as siderophores that can bind with iron-binding proteins of the host [13, 14]. The synthesized and secreted siderophores are capable of binding to ferric iron with an affinity about 10 times higher than transferrin or lactoferrin. Under different conditions of the host body, *K. pneumoniae* can synthesize various species of siderophores, and the most important type is Enterobactin, often known as Enterochelin. The siderophore seems to be the largest iron uptake system synthesized by this bacterium [15]. Other siderophores produced by *K. pneumoniae* include aerobactin, ferrichrome, ferric citrate, salmochelin, yersiniabactin, heme, ferric iron and siderophores necessary for ferrous (two systems) uptake. Ten iron uptake systems have been known so far in *K. pneumoniae*; however, six iron uptake systems have been observed in non-pathogenic species of *K. pneumoniae* and ten iron uptake systems in invasive isolates, especially isolates producing liver abscesses [11].

The genes encoding iron uptake system in *K. pneumoniae* include *fhuA* (ferrichrome), *iucABCD* and *iutA* (aerobactin), *fepAB* and *entAB* (enterobactin), *fecAE* (ferric citrate), *iroNB* (salmochelin), *hmuR* (heme), *sitA* and *feo* (ferrous, Fe²⁺), *kfuABC* (ferric, Fe³⁺), *fyuA* and *irp-1*, *irp-2*, *ybtS* and *yersiniaHPI* (yersiniabactin) [16, 17]. Despite the epidemiological impor-

tance of *K. pneumoniae*, limited studies have been carried out on the importance of iron uptake systems in the pathogenicity of this species. Considering the importance of the iron uptake system, behind the bacterial capsule structure, as a virulence factor in *K. pneumoniae*, and due to the significance of diseases caused by *K. pneumoniae* as an opportunistic pathogen, the present study compared the genes involved in iron uptake and their prevalence among environmental and clinical isolates of *K. pneumoniae* aiming to determine the active role of these genes in the pathogenicity of this bacterium.

2. MATERIALS AND METHODS

2.1. Organism Collection

A total of 150 clinical and 150 environmental isolates were involved in this study. Clinical isolates were collected through October.2014 – December.2016 from Ilam hospitals and Clinical Laboratories. Environmental isolates were gathered through April 2015- December.2016 from the sewage of Ilam. In 30 times of sampling, 600 samples were collected from sewage. With regard to the fluency of sewage, sampling was performed within 5 minutes. The volume of sewage was almost 1.5 liter in every/each sampling. To avoid collecting the same source of bacteria, sampling from sewage was performed twice a month. All clinical and environmental samples were identified by biochemical tests such as Simon citrate, TSI, SH2, SIM, MR-VP, Urease, and Lysine Iron Agar.

The current study was performed based on the ethical standards Declaration of Helsinki and all individuals provided written informed consent that was approved by the Ethics Committee of The Ilam University of Medical Sciences

2.2. DNA Extraction

The suspension was prepared by LB broth and incubated at 37°C for 18-24h. The samples were then centrifuged at 3500 rpm for 10 min and the pellet used for extraction. PCR was done to investigate *iucD*, *iroN*, *kfuA*, *hmuR* and *yHPI*. Primer is mentioned in Table 1. PCR reaction was in 25µl and 3µl of primers added to Master Mix.

2.3. Statistical Analysis

Data analysis was performed by using SPSS software version 19.0 for windows [IBM, Chicago, IL, USA]. A chi-square test was used to compare the prevalence of iron uptake coding genes among clinical and environmental isolates of *Klebsiella pneumoniae*. A p-value < 0.05 was considered as statistically significant.

3. RESULTS

The frequency of *kfuA*, *iroN*, *iucD* and *yHPI* among clinical isolates were 33.3%, 24.7%, 16.7%, and 15.3% respectively, additionally, the frequency of these genes among environmental isolates was 20.7%, 49.3%, 6%, and 0.7%, respectively. *hmuR* was neither detected in clinical nor in environmental isolates (Table 2). As indicated in Table 2, there is a significant difference between the frequency of *kfuA* gene two groups of Study (P=0.016). In this study, it was found that some isolates had more than one iron uptake-related gene.

Based on the results of identifying the genes, 18.6% (28 isolates) of the clinical isolates and 9.33% of the environmental isolates had two iron uptake-related genes and 6% among the clinical isolates, and 2% of the environmental isolates had three iron uptake-related genes. In addition, 35.33% of the clinical samples and 46.66% of the clinical samples had only one iron uptake-related gene (Table 2).

In this study, the clinical isolates with two iron uptake-related genes were classified into six groups, including aerobactin-yersiniabactin-positive isolates (n=2), salmochelin-aerobactin-positive isolate (n=1), salmochelin-ferric iron-positive isolates (n=10), ferric iron – Yersiniabactin-positive isolates (n=2), aerobactin - ferric iron-positive isolates (n=9) and yersiniabactin – salmochelin-positive isolates (n=4). The environmental isolates were classified into three groups, including salmochelin - ferric iron isolates (n=11), salmochelin - aerobactin isolates (n=2) and salmochelin - yersiniabactin isolate (n=1). In the clinical isolates with three iron uptake-related genes, there were three groups, including salmochelin-yersiniabactin-ferric-iron-positive isolates (n=5), salmochelin-aerobactin-ferric-iron-positive isolates (n=3) and aerobactin-yersiniabactin-ferric iron-positive isolate (n=1). The environmental isolates had only one group including salmochelin- yersiniabactin-ferric-iron-positive isolates (n=3)(Suppl. Figs.1-4).

4. DISCUSSION

Undoubtedly, the role of *K. pneumoniae* is clear in causing

serious infections in people with immunodeficiency and diseases such as those with diabetes mellitus and alcoholics. In recent years, there have been reports of the occurrence of community-acquired liver abscesses in people with a healthy immune system by pathogenic subspecies of *K. pneumoniae* in Taiwan and some other Asian countries [18]. This syndrome is associated with dissemination into the eye, meninges, brain and other organs. There is a theory that the presence of iron uptake-related genes are effective in increasing the pathogenicity of the less virulent *K. pneumoniae* subspecies and the presence of these genes may be the reason for the increased pathogenicity of *K. pneumoniae* [18, 19]. Although *K. pneumoniae* is epidemiologically one of the most important species in the Enterobacteriaceae family, unfortunately, little *in vivo* studies have been conducted on the importance of iron uptake systems in bacterial growth, virulence and the duration of *K. pneumoniae* infection as compared to other bacterial pathogens [20]. Considering the importance of the iron uptake system in the pathogenesis of *K. pneumoniae*, this study compared the frequency of genes encoding proteins involved in iron uptake and their prevalence among environmental and clinical strains of *K. pneumoniae*, which we will discuss below in the results of the studies.

This study examined 300 samples [150 clinical and 150 environmental samples] of *K. pneumoniae* for the presence of iron uptake-related genes. The clinical samples were urine specimens and more than 90% of environmental samples were isolated from urban sewage.

Table 1. Details of recruited Primers.

Primer Name	Primer Sequence	Annealing Temperature (°C)	Length of PCR Product (bp)
<i>Kfu A</i>	5-GAAGTGACGCTGTTTCTGGC-3 5-TTTCGTGTGTGGCCAGTGACTC-3	58.7	799
<i>iucD</i>	5-TACAGACCGACCTCCGTGAA-3 5-CTGCGAGGGCTGACACATTA-3	58.7	203
<i>hmuR</i>	5-AAAAACCGCTATGGGCGAGA-3 5-GAAAGTGAAAACCGCACCC-3	57.7	417
<i>iroN</i>	5- GAATGAACTACCGCCCCA-3 5-AGATACTCGCGGCATGTCAG-3	58.7	202
<i>yHPI</i>	5-GAAGTGACGCTGTTTCTGGC-3 5-TTTCGTGTGTGGCCAGTGACTC-3	57.7	620

Table 2. Frequency of iron uptake genes among the clinical and environmental isolates of *K. pneumoniae*.

<i>hmuR</i>		<i>kfuA</i>		<i>yHPI</i>		<i>iroN</i>		<i>iucD</i>		Gene
								Sample source ¹		
Env	Clin	Env	Clin	Env	Clin	Env	Clin	Env	Clin	Frequency
0	0	31 [20.7]	50 [33.3]	1 [0.7]	23 [15.3]	74 [49.3]	37 [24.7]	9 [6]	25 [16.7]	Positive [%]
150 [100]	150 [100]	119 [79.3]	100 [66.7]	149 [99.3]	127 [84.7]	76 [50.7]	113 [57.3]	141 [94]	125 [83.3]	Negative (%)
0		0.016		0.847		0.077		0.173		P value

1, Samples were provided from Environmental [Env] and Clinical [Clin] source

In this study, the frequency of *kfuA*, *iucD*, *iroN*, and *yHPI* genes were 33.3%, 16.7%, 24.7% and 15.3% in the clinical isolates, as well as 20.7%, 6%, 49.3% and 0.7% in the environmental samples, respectively. The highest frequency in the clinical isolates was related to *kfu* gene (n=50) followed by *iroN* (n=37), *iucD* (25n =) and *yHPI* (n=23) genes, respectively, and the highest frequency in the environmental isolates was related to the *iroN* gene (N=74), followed by *kfu* (n=31), *iucD* (n=9) and *yHPI* (nn=1) genes, respectively, but none of the clinical and environmental isolates of *K. pneumoniae* had *hmuR* gene.

Frequency distribution of the desired genes involved in the iron uptake system in the clinical isolates of *K. pneumoniae* cannot be important in terms of gender due to the disparity between the number of isolates among men (38%) and women (62%).

Considering that the presence of *kfuA* gene in the clinical isolates was more than that of in the environmental isolates, based on the statistical analysis and considering P values <0.05, this difference was statistically significant (P=0.016). However, despite the high prevalence of the *iroN* gene in the environmental isolates compared to the clinical isolates, this difference was not statistically significant (P=0.077). In addition, due to the higher prevalence of *iucD* and *yHPI* genes among the clinical isolates, there was no statistically significant difference between the presence of *iucD* (p=0.173) and *yHPI* (P=0.847) genes in the clinical and the environmental isolates.

Huang *et al.* (2012) in Taiwan introduced the iron uptake system as one of the most important virulence factors in *K. pneumoniae*. In this study, 34 clinical isolates of *K. pneumoniae* from liver abscesses had enterobactin (100%), aerobactin (50%), ferric iron (30%), heme (100%) and yersiniabactin (8%) genes [21].

Aher *et al.* (2012) examined the frequency of *kfu* gene among eight isolates of *K. pneumoniae* isolated from the nose swab and tissue samples, which was reported to be 25% [22]. The *Kfu* gene may play a major role in bacterial pathogenicity. This operon enables the bacterium to iron uptake even at concentrations of the host body. In our study, the *kfu* gene had the highest frequency (33.3%) among the clinical specimens and the second place among the environmental samples with a frequency of 20.7%. In a study of Fung in 2012, all 10 strains of *K. pneumoniae* isolated from the liver abscesses had *Diuc* [aerobactin], *hmuR* [heme], and *iroN* [salmochelin] genes [23]. The *K. pneumoniae* strains encode the enterobactin, aerobactin, yersiniabactin and salmochelin genes variably. Invasive strains, unlike classical strains, use increasingly salmochelin, aerobactin, ferric iron and yersiniabactin systems. It has been found that these genes are effective in maintaining the infection in the host and also developing a more pathogenic phenotype in *K. pneumoniae*.

According to the studies, the strains of aerobactin-producing *K. pneumoniae* had a higher virulence compared to non-producing strains. In addition, these strains are capable of developing acute community-acquired infections [18].

In our study, the frequency of aerobactin and yersiniabactin genes in the clinical samples of *K. pneumoniae*

was nearly 7-18%, as most studies in the world. In some studies, such as a study by Chang *et al.* on 34 clinical isolates of *K. pneumoniae*, the frequency of these genes was reported to be 50%. However, the frequency of this gene was lower in the environmental samples, probably confirming the role of these genes in the pathogenicity of *K. pneumoniae*.

The notable difference in the frequency of iron uptake-related genes between this study and others in the world is the isolation site of samples. In this study, as mentioned, all clinical samples were isolated from urine, while similar studies were mostly performed on the samples isolated from liver and blood abscesses. The nosocomial infections with *K. pneumoniae* are often involved in urinary and respiratory systems. Since these two organs of the body are in relation to various host defense mechanisms, the virulence factors in the UTI pathogens seem to be different from the strains found in the respiratory tract isolated from the patients with *K. pneumoniae*.

In this study, it was found that some isolates had more than one iron uptake-related gene. Based on the results of identifying the studied genes, 18.6% [28 isolates] of the clinical isolates and 9.33% of the environmental isolates had two iron uptake-related genes and 6% of the clinical isolates and 2% of the environmental isolates had three iron uptake-related genes. Moreover, 35.33% of the clinical samples and 46.66% of the clinical samples had only one iron uptake-related gene. In this study, the clinical isolates with two iron uptake-related genes were classified into six groups, including aerobactin-yersiniabactin-positive isolates (n=2), salmochelin-aerobactin-positive isolate (n=1), salmochelin-ferric iron-positive isolates (n=10), ferric iron – Yersiniabactin-positive isolates (n=2), aerobactin - ferric iron-positive isolates (n=9) and yersiniabactin – salmochelin-positive isolates (n=4). The environmental isolates were classified into three groups, including salmochelin - ferric iron isolates (n=11), salmochelin - aerobactin isolates (n=2) and salmochelin - yersiniabactin isolate (n=1). In the clinical isolates with three iron uptake-related genes, there were three groups, including salmochelin-yersiniabactin-ferric-iron-positive isolates (n=5), salmochelin-aerobactin-ferric-iron-positive isolates (n=3) and aerobactin-yersiniabactin-ferric iron-positive isolate (n=1). The environmental isolates had only one group including salmochelin-yersiniabactin-ferric-iron-positive isolates (n=3). Although 41.01% of the environmental isolates and 40.07% of the clinical isolates used none of the iron uptake genes examined in this research, the role of these iron uptake-related genes in the pathogenicity of this bacterium could not be ruled out because of failing to evaluate all genes involved in iron uptake in *K. pneumoniae* in this study, and that this group of *K. pneumoniae* isolates might have used other iron uptake-related genes.

In a study conducted by Bachman *et al.* (2011) in the United States on the siderophores expressed by 129 clinical isolates of *K. pneumoniae*, three groups of the Klebsiella strains were identified including the isolates positive for enterobactin (81%), enterobactin-yersiniabactin (17%), enterobactin-salmochelin [glycosylated enterobactin] with or without yersiniabactin (2%). In this study, the highest

prevalence of isolates positive for enterobactin and yersiniabactin was found among the respiratory and β -lactam-resistant isolates and the lowest prevalence in urinary tract isolates, indicating the unnecessary expression of these siderophores in the urinary tract [24].

CONCLUSION

The results of this study can indicate the importance of the ferric iron system in the iron uptake from the host body and the salmochelin system in the iron uptake from the environment. In this study, it was found that the frequency of genes encoding proteins involved in the iron uptake varies between clinical and environmental isolates of *K. pneumoniae*. The host body conditions and iron availability seem to be effective in the level of using these systems. The results of this study, due to the difference in iron uptake-related genes between clinical and environmental isolates of *K. pneumoniae*, indicated the significance of iron uptake systems in the virulence of the bacteria. It should also be noted that due to the difference between the isolation sites of the samples in this and other studies, it can be concluded that the hypervirulent and tissue-invasive strains of *K. pneumoniae* employ further iron uptake systems.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by The Ilam University of Medical Sciences, Iran.

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. All human procedures were followed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the *Helsinki Declaration* of 1975, as revised in 2008 (<http://www.wma.net/en/20activities/10ethics/10helsinki/>).

CONSENT FOR PUBLICATION

Informed consent was obtained from each participants.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [NS], upon reasonable request.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers website along with the published article.

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