

Molecular and phenotypic detection of siderophore production by hypervirulent *Klebsiella pneumoniae*



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ABSTRACT

Klebsiella pneumoniae is a highly prevalent pathogen among nosocomial and community-acquired infections, including urinary tract infections (UTIs), pneumonia, surgical site infections, and bloodstream infections.

One hundred (100) isolates were obtained from different sources, including urine, burns, sputum, blood, and wound. All isolates were bacteriologically identified as *Klebsiella pneumoniae* using phenotypic and biochemical tests. The antimicrobial susceptibility test was done by using the disc diffusion method for thirteen different antimicrobial agents. String test was used to evaluate the hypermucoviscosity of the isolates and siderophore production was estimated by CAS agar and colorimetric methods. Two genes, *iucA* and *iroB*, were detected by PCR and their correlation with phenotypic markers was evaluated. The results showed that 51% of the isolates were positive for the string test and 49 % were negative. The results showed that the siderophores production ranged between 14 to 74 psu. Genotyping results showed that 23% of the isolates contained *iucA* gene and 17 % contained *iroB*. There was a high correlation between the two genes and the hypermucoviscosity. Also, siderophore production was highly associated with having one of the genes or both with more than 60 psu of siderophores production.

INTRODUCTION:

Klebsiella pneumoniae is a highly prevalent pathogen among nosocomial and community-acquired infections, including urinary tract infections (UTIs), pneumonia, surgical site infections, and bloodstream infections. It is the second most frequent cause of gram-negative sepsis [1-3]. Several virulence factors are present in *K. pneumoniae*, including capsular polysaccharide (CPS) and a crucial virulence component that is a significant factor in developing sepsis [4,5]. Two distinct groups of clinical isolates of *Klebsiella pneumoniae* (two pathotypes) were discovered. Classical strains (cKp) are typically isolated from patients with some degree of immunocompromise, and that known as carbapenem-resistant (CR). In contrast, hypervirulent strains (hvKp) are associated with community-acquired invasive infections, and those are sensitive to carbapenem [6,7].

The string test is a routine and commonly used microbiological test to identify hypermucoviscosity (HMV). Hypermucoviscosity phenotype is used to identify the hypervirulent species of *K. pneumoniae*. The hypermucoviscous phenotype is conferred by the hypercapsule produced by hypervirulent *K. pneumoniae* (hvKP) [8]. CPS inhibits the expression of human defense mechanisms as well as neutrophil and macrophage phagocytosis [9]. Moreover, the hypercapsule strengthens protection against numerous humoral defensive systems [8]. Because of this, hvKP is less susceptible to complement killing than classical *K. pneumoniae* (cKP) [10]. Low-molecular-weight secondary metabolites called siderophores have the ability to chelate iron. These are substances with tiny peptide molecules having side chains and functional groups that have great affinity ligands for binding to ferric ions and transporting them across the cell membrane [11]. Even at deficient concentrations, microbial siderophores effectively chelate iron and stimulate its absorption by forming a ferric-siderophore complex [12]. Siderophores are also necessary factors for

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K. pneumoniae pathogenesis [6]. *K. pneumoniae* needs a scarce amount of iron from the environment to survive during infection, which is not easily accessible in the host. This is mainly because the host sequesters it as part of the nonspecific immune response to limit the proliferation of several potential pathogens. Typically, there is often little free iron in host plasma because it is joined by iron transport molecules like transferrin. Mammalian hosts can lower iron levels during bacterial infection by switching iron binding to lactoferrin, an inherent defense protein found in body fluids [13-15]. The dominant mechanism used by many pathogens, including *K. pneumoniae*, to gain iron is by the production of siderophores, which are substances with a greater affinity for iron than host transport proteins do. Iron can be scavenged from the environment or from the host's iron-chelating proteins by siderophores [15]. *Klebsiella pneumoniae* expresses several siderophores, including enterobactin, yersiniabactin, salmochelin, and aerobactin [16]. Salmochelin, a specific kind of siderophore encoded by the *iroB* gene, is only present in around 2 to 4% of nosocomial *K. pneumoniae* strains (CKP). It is 90% more common in hypervirulent *K. pneumoniae* strains [17,18]. Aerobactin is a specific kind of siderophore encoded by the *iucA* gene [19]. Classical nosocomial *K. pneumoniae* clinical isolates seldom express it, but hypervirulent *K. pneumoniae* isolates do [20,21]. On the other hand, not all hyper-capsulated strains possess this siderophore gene [20]. Hypervirulent *K. pneumoniae* strains can acquire iron using several siderophore genes, and aerobactin gene is one of the most genes used in at least one hvKp strain [21]. The siderophore production is commonly detected using the chrome azurol sulphonate (CAS) assay as mentioned by Schwyn and Neilands [22]. Additionally, Solid CAS agar media are also commonly known that is used to detect siderophore production in hypervirulent species [23].

MATERIAL AND METHODS

Sample Collection

Patients suspected with *Klebsiella* infections were subjected to this study. Isolates were collected from patients who attended four main hospitals located in Al-Anbar province, western Iraq, between June and October 2022. All isolates were isolated from urine, burns, sputum, blood, and wounds.

Isolates identification

Traditional and biochemical tests were used to identify all *Klebsiella pneumoniae* isolates by using cultural methods, microscopic identification, and biochemical tests. MacConkey agar and Blood agar were used to grow and identify all isolates. Gram stain, indole, Methyl Red, Voges-Proskauer, Citrate utilization, catalase, oxidase, and urease were used to identify *Klebsiella* isolates according to Mahon and Lehman [24]. All isolates were confirmed automatically at the species level by using VITEK-2 compact system with identification of G-ve bacteria (ID-GNB) cards following the manufacturer's instructions.

Antibiotics Susceptibility Tests

The Kirby-Bauer method was followed as described by CLSI (2021) to perform the antibiotics susceptibility test (Table 1).

String test

To be considered hypermucoviscous (HMV), bacterial isolates were cultured on blood agar and incubated at 37°C for 24 h. A sterilized loop was used to stretch the mucoid of the colony, 5 mm or more of the viscous filament from the colony's surface was accounted as HMV. The test was used to differentiate between hvKp and cKp phenotypically [19].

Siderophore estimation assay

The Chrome Azurol Sulphonate (CAS) assay was used to test the siderophore-production capacity of *Klebsiella* isolates [22]. All glassware were first cleaned with 3 mol/l hydrochloric acid (HCl) to remove iron, then rinsed in deionized water. A quantitative method was used to calculate the amount of the production of the siderophore, CAS reagent was made following Louden *et al.* [25].

Chrome Azurol Sulphonate reagent (blue dye CAS)

The blue dye was used to estimate siderophore production and was prepared as follows: Solution (1) was prepared by diluting 0.06 g of CAS (Fluka Chemicals) with 50 ml of ddH₂O. Solution (2) was prepared by mixing 0.0027 g of FeCl₃-6 H₂O with 10 ml of 10 mM HCl. Solution (3) was prepared by mixing 0.073 g of hexadecyltrimethylammonium bromide (HDTMA)

Table 1. Antimicrobial agents used in this study.

Class	Antibiotic	Abbr.	Conc.
Aminoglycosides	Gentamicin	GM	30 µg
	Amikacin	AK	30 µg
Penicillins(β-lactamase)	Amox- Clavu	AMC	20/10 µg
	Piper-tazoba	TPZ	100/10 µg
Folatepathway inhibitors	Trim- sulpha	SXT	1.25/23.75 µg
Cephalosporins	Ceftriaxone	CRO	30 µg
	Cefotaxime	CTX	30 µg
	Cefepime	FEP	30 µg
	Ceftazidime	CAZ	30 µg
Carbapenem	Imipenem	IPM	10 µg
Monobactam	Aztreonam	ATM	30 µg
Quinolones	Levofloxacin	LEV	5 µg
	Ciprofloxacin	CIP	5 µg

with 40 ml of ddH₂O. Solution (1) was combined with 9 ml of solution (2) and then mixed with Solution (3). The mixture should be blue after mixing, and then it was autoclaved in a polycarbonate bottle, stored in a plastic container/bottle.

Chrome Azurol Sulphonate agar plate

Klebsiella pneumoniae pathogenicity may be accurately assessed using a quantitative siderophore production test, which may also help differentiate hvKp from cKp isolates. For 1 L of CAS agar, 900 ml of Laurie Bertani agar was prepared and autoclaved. The medium was cooled to 60 °C, and 100 mL of the blue dye (CAS solution) was added with stirring. The final medium was aseptically poured into Petri dishes. Sterilized filter paper discs (5 mm diameter) were aseptically fixed on the plates. An inoculum (10µL) of overnight *Klebsiella pneumoniae* isolates (OD₆₀₀=0.5) was pipetted into the paper disc. The CAS agar plates were incubated at 37°C for 24 hours, and the control plates were prepared similarly without bacterial inoculum. The size of an orange halo zone around the paper disc that indicates the siderophore production was measured by millimeter. The optimal siderophore production cut-off point to predict hvKp was determined at 9.6 mm [26].

Analysis of siderophore production using colorimetric methods

Quantitative estimation of siderophore production was performed using colorimetric techniques.

Traditional method

Bacterial cultures cultivated in an LB broth medium were used, and their supernatants were utilized to quantitatively determine siderophore [27].

In a 1.5 ml Eppendorf tube (containing 1 ml of LB broth), 10 µl of a freshly grown bacterial culture (10⁸ cfu/ml) was added to the tube and incubated for 24 hours at 37 °C. The control was prepared similarly without any inoculum. Then, the tube was centrifuged at 10,000 rpm for 10 min, and the supernatant was then used to calculate the amount of siderophore. Each bacterial culture's supernatant (0.5 ml) was combined with 0.5 ml of CAS reagent, and after 20 minutes, optical density at 630 nm was measured. The amount of siderophore was calculated in Percent Siderophore Units (psu) [28], which was determined by using the formula:

$$\text{Siderophore production (psu)} = \frac{Ar-As}{Ar} * 100$$

Where Ar = reference absorbance (CAS solution and un-inoculated broth), and As = the sample's absorbance (CAS solution and cell-free supernatant of sample).

Modified microplate assay

The modified method was done by using 96 well microtiter plate. In a microcentrifuge tube, 0.5 ml of colonized (5 µL inoculum containing 10⁸ cfu/ml) broth was used to obtain the supernatant. In each microplate well, 100 µL of the supernatant from each bacterial culture was added, then the 100µL CAS reagent was added. Following the incubation, the optical densities were measured at 630 nm using a microplate reader, and then the siderophore was estimated using the formula mentioned above [29].

Chrome Azurol Sulphonate broth and Chrome Azurol Sulphonate agar (CAS media)

CAS agar was prepared by mixing 100 ml CAS reagent in 900 ml sterilized LB agar medium. CAS reagent solution was autoclaved in a polycarbonate bottle or turbid white tube, which was washed with HCl and then rinsed with distilled water [26,29].

Molecular markers

Two genes, *iucA* and *iroB*, were selected to differentiate hvKp. The genomic DNA of *Klebsiella* isolates was extracted using Wizard® Genomic DNA

Purification Kit, Promega (USA), following the manufacturer’s instructions. PCR analysis was performed using the Applied Biosystems Bio-Rad instrument (UK). *iucA* was amplified using the primers F-GCTTATTTCTCCCAACCC and R-TCAGCCCTTTAGCGACAAG, and *iroB* was amplified using the primers F-ATCTCATCATCTACCCTCCGCTC and R-GGTTGCGCCGTCGTTTTCAA [19].

PCR-based detection of these genes used (per reaction) 10 µL of 2x GoTaq® Hot Start Green Master Mix (Promega /USA), 1 µL of forward primer, 1 µL of reverse primer (10µmol/µL), 60 ng/µL of gDNA, and the volume was adjusted to 20 µL using free nuclease water. PCR cycling conditions were followed: initial denaturation at 95 °C for 3 min, (denaturation at 95 °C for 30 s, annealing at 56 °C for 35 s, extension at 72°C for 1 min) for 30 cycles, and final extension at 72.0°C for 10 min. All PCR products were analyzed using 1.5% agarose.

RESULTS

Identification of Klebsiella isolates

One hundred *Klebsiella* isolates were isolated from four hospitals in Al-Anbar province and were identified using cultural and biochemical tests and confirmed by VITEK-2 compact system. Out of the 100 isolates, 44 were obtained from urine samples, 23 from burns samples, 19 from sputum, 9 from blood, and 5 from wounds, Table 2.

Differentiation of hvkp using String test

The hypermucoviscosity is defined by the string test, and the isolate is identified as hvKp when a viscous filament stretches 5mm or more from the colony surface [19], Figure 1. The results showed that 51% of the isolates were positive for the string test and defined as a hvKp and 49% were negative for the string test and defined as cKp. String test results showed that 100% of the Blood isolates were positive, sputum isolates (78.9%), wounds (60%), urine (36.3%), and burns (34.7%), as shown in Table 2.

Table 2. Percentage of a positive string test.

Site	Number	Positive string	Negative string
Urine	44	16 (36.36 %)	28(63.64%)
Burns	23	8 (34.78 %)	15(65.22%)
Sputum	19	15 (78.95 %)	4(21.05%)
Blood	9	9 (100 %)	0
Wound	5	3 (60 %)	2(40%)

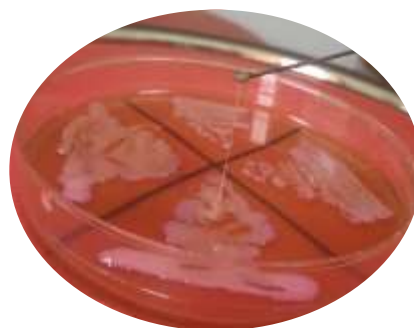


Figure 1. Positive string test. Shows the viscous filament length

Antimicrobial resistance profile

The 100 isolates showed varying degrees of resistance. Both pathotypes (hvKp and cKp) were quietly similar in their resistance profile toward antibiotics used in this study. hvKp and cKp showed a high degree of resistance against Amoxicillin-Claculanic acid, Trimethoprime-Sulphamethaxazone, Ceftazidime, Ceftriaxone, and Cefotaxime as shown in Table 3. cKp isolates were more resistance to Imipenem (6.12%). In contrast, hvKp isolates were all sensitive to the same antibiotic.

Chrome azurol S agar plate assay (CAS)

A zone of orange color surrounding the colonies created by bacteria cultured on CAS indicates siderophores production, as shown in Figure 2. The siderophore production was roughly calculated based on the halo size developed on CAS agar around the colonies. The ideal siderophore production cut-off point for an isolate to be considered hvKp strain was 9.6 mm. The results showed that 51 (51%) of the 100 K. pneumonia clinical isolates were hvkp, as the orange halo around the colonies ranged between 9.8-27 mm in diameter, as shown in Figure 3.

Antibiotic	hvKp (51)	cKp (49)
	Resistance	
Amox-Clavulanic acid	51 (100%)	47 (95.92%)
Cefotaxime	44 (86.27%)	46 (91.84%)
Piperaci-tazobactam	28 (54.90%)	25 (51.02%)
Trimeth. - sulphameth.	47 (92.16%)	46 (91.84%)
Ceftriaxone	44 (86.27%)	46 (93.88%)
Ceftazidime	50 (98.04%)	49 (100%)
Cefepime	37 (72.55%)	37 (75.51%)
Imipenem	0	3 (6.12%)
Aztreonam	29 (56.86%)	24 (48.98%)
Amikacin	16 (31.37%)	12 (24.49%)
Gentamicin	29 (56.86%)	32 (65.31%)
Levofloxacin	31 (60.78%)	26 (51.02%)
Ciprofloxacin	34 (66.67%)	30 (61.22%)

Table 3. Antimicrobial-resistant profile of the two pathotypes

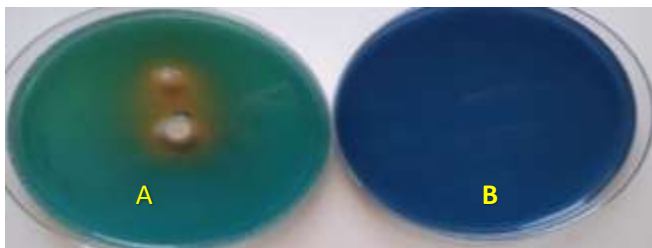


Figure 2. (A) The orange halo zone due to siderophore production, (B) the CAS agar plate’s control.

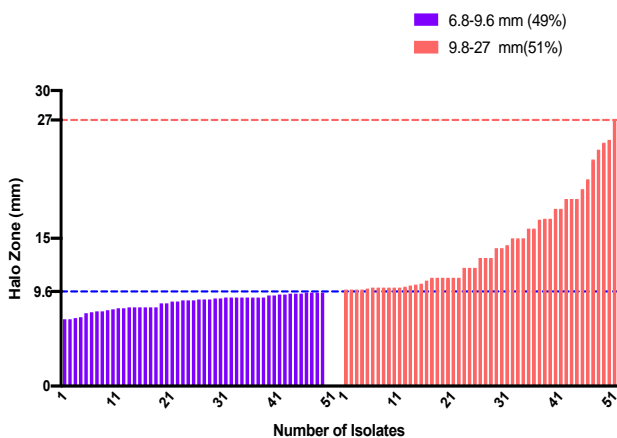


Figure 3. The percentage of hvKp and cKp using the CAS method.

Colorimetric methods

The CAS agar method is not optimal and can only give an approximate estimation of the siderophore production. A modified method and CAS reagent were used to quantify the siderophore production for more accurate results. As mentioned in the Methods, two phenotypic methods were used to estimate the siderophore production accurately.

The amount of the siderophore produced by all 100 isolates was checked using the proposed traditional and modified microplate methods. The level of siderophore produced by bacterial isolates ranged from 14 to 74 psu when measured by the microplate technique. The results indicated that 56% of the isolates showed siderophore production ranged between 30-74 psu, and 44% were less than 30 psu producer, as shown in Figure4.

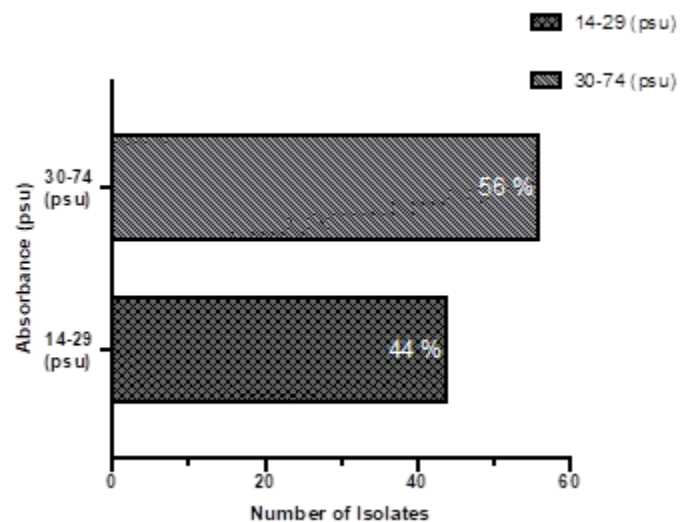


Figure 4. Estimation of siderophores production using colorimetric methods.

Detection of *iroB* and *iucA* genes

PCR results showed that the *iucA* gene was found in 23 isolates (23%) and *iroB* in 17 isolates (17%), Figure 5. The two genes were detected in the same isolate in 10 out of the 100 *Klebsiella* isolates.

The PCR and string test results indicated that out of the 23 isolates carrying *iucA*, 19 isolates (82.6 %) were positive for the string test, and 15 isolates (88.2%) of the 17 isolates carrying *iroB* were positive for the string test. Interestingly, all 10 isolates carrying both genes were 100% positive for the string test.

The siderophore production of all 23 isolates carrying *iucA* ranged between 32-74 psu. All isolates carrying *iroB* were very high producers ranging between 64- 74 psu, as shown in Table 4. The distribution of isolates according to the site of isolation is indicated in Table 5. *iucA* and *iroB* were most prevalent in blood sample isolates, with 88.89% and 66.6% for *iucA* and *iroB*, respectively.

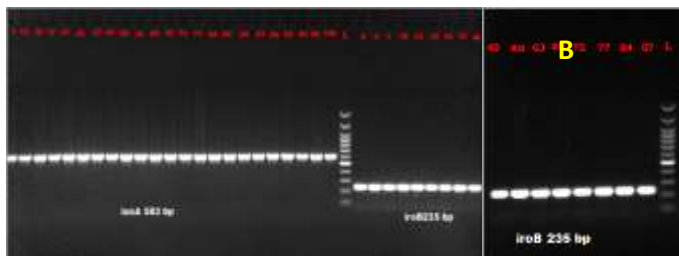


Figure 5. Gel electrophoresis of the PCR products. (L) ladder 100 to 1200 bp, (A) left of the ladder *iucA* bands 583, (A) right of the ladder and (B) *iroB* bands 235 bp.

Table 4. Distribution of *iucA* and *iroB* and their association with phenotypic markers

	<i>iucA</i> (23)	<i>iroB</i> (17)	<i>iucA</i> + <i>iroB</i> (10)
String test (51)			
positive	19 (82.61%)	15 (88.24%)	10 (100%)
negative	4 (17.39%)	2 (11.76%)	0
<i>P</i> value	0.0007	0.0009	0.0012
Siderophore production (56)			
>30 psu	23 (100%)	17 (100%)	10 (100%)
<30 psu	0	0	0
<i>P</i> value	<0.0001	<0.0001	0.0022

Table 5. Distribution of molecular markers among sources of infection.

Source	<i>iucA</i>	<i>iroB</i>	<i>iucA</i> and <i>iroB</i>
Urine (44)	7(15.91%)	7(15.91%)	3(6.82%)
Burns (23)	4(17.39%)	2(8.70%)	1(4.34%)
Sputum (19)	4(21.05%)	1(5.26%)	1(5.26%)
Blood (9)	8(88.89%)	6(66.67%)	5(55.56%)
Wound (5)	0	1(20%)	0

DISCUSSION

Out of the 100 *K. pneumoniae* isolates, 51(51%) hvKp strains were identified using string tests. The percentage is differed according to the region and the source of isolation. The antibiotic resistance patterns are caused by various factors, including the number of samples, regional differences, the source of specimens, the site of infection, and patient predilection [30]. Other studies indicated that a string test identified 33% and 45.7% of *K. pneumoniae* isolates as hypermucoviscous (hvKp) [31,32]. Blood isolates had a higher percentage of hypermucoviscous strains (100%), followed by sputum isolates. Hypermucoviscous is an essential virulence factor in causing sepsis [5].

Hypervirulent *Klebsiella pneumoniae* isolates have evolved and have been of major clinical concern for the past 40 years after identifying this pathotype. The results showed that hvKp isolates were resistant to all antimicrobial agents used in this study except for Imipenem, Table 3. This observation is attributable to hvKp isolates acquiring chromosomal or plasmid-encoded antibiotic resistance genes found on mobile genetic elements like plasmids and ICE [33]. However, compared to the cKp strains in this investigation, a considerably higher number of hvkp strains showed resistance to the tested antimicrobials such as Amoxicillin- Clavulanic acid, Piperacillin-tazobactam, Trimethoprim- sulphamethoxazole, Aztreonam, Amikacin, and Levofloxacin which is consistent with Wang *et al.* [34]. The cKp isolates appeared more resistant to other antimicrobial agents such as Cefotaxime, Ceftriaxone, Ceftazidime, Cefepime, Imipenem, and Gentamicin. The results of the resistance profile between the two pathotypes are consistent with another research [31]. These findings support the idea that hvKp strains are developing an increased level of antibiotic resistance that was changed over time.

It is obvious that the genotype and phenotype of hvKp have been modified, but it is unclear what caused this modification. Classical *Klebsiella* (cKp) strains seem genetically different from hvKp despite having certain virulence traits in common [35,36]. Initially, it was suggested that the distinguishing features of hvKp isolates are defined by a hypermucoviscous phenotype [37]. This study showed an association between having *iucA* and *iroB* genes with string tests that were

statistically highly significant, Table 4. This indicates an impact of iron on cps expression. Fur and IscR are iron-responsive transcriptional regulators in the Enterobacteriaceae that regulate cps. iscR activated the cps to increase capsule production when there is a sufficient amount of iron.

In contrast, fur indirectly suppresses cps when there is a high concentration of iron and relieves this effect when the iron level is low [38]. Absorption of ferric iron, which is more common in hvKP strains than cKP strains, is necessary for maximal systemic virulence of hvKp isolates. These results align with a study done in Sudan [39].

Siderophores production was assessed using two techniques as mentioned in the methods. Isolates that contain iron acquisition genes appear to produce more siderophore than others. In this study, *iucA* and *iroB* genes were highly correlated with siderophores production in *Klebsiella* isolates, Table 4. All isolates with one of the genes or both showed high production of siderophores with more than 30 psu compared to other isolates lacking any of the two genes. This association indicates the importance of *iucA* and *iroB* in iron acquisition and siderophores production. The results agree with previous studies that demonstrated that hvKp produces high siderophore than cKp due to the presence of these two genes that have a great affinity for iron (19, 40- 42).

CONCLUSION

The phenotypic and molecular biomarkers identification of local *K. pneumoniae* isolates revealed a high correlation between having *iucA* and *iroB* genes with the hypermucoviscosity and siderophores production. *Klebsiella*'s two phenotypes are regulated as both are connected through iron acquiring by siderophores chelating agent. Our findings highlighted the necessity to periodically monitor *Klebsiella* infections to decrease the spread of hvKp strains that could increase in our region. Further investigations are needed to identify the severity of the public health risk raised by hvKp strains in Iraq.

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الكشف الجزيئي والمظهري لانتاج Siderophores للكليسيلا الرئوية عالية الضراوة

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الخلاصة :

بكتريا الكليسيلا الرئوية هي مُمرضٌ منتشر بشكل كبير بين عدوى المستشفيات ومسبب لكثير من الالتهابات بين الناس الاصحاء، بما في ذلك التهابات المسالك البولية (UTIs) والالتهاب الرئوي والتهابات الجروح والتهابات مجرى الدم. تم الحصول على ١٠٠ عزلة من مصادر مختلفة منها الادرار والحروق والبلغم والدم والجروح. تم تشخيص جميع العزلات على أنها *Klebsiella pneumoniae* باستخدام الاختبارات المظهرية والاختبارات البايوكيميائية وتم تأكيد التشخيص باستخدام VITEK-2. تم إجراء اختبار الحساسية للمضادات الحيوية باستخدام طريقة الانتشار بالاقراص باستخدام ثلاثة عشر مضاد حيوي. استخدم اختبار String test لتقييم اللزوجة في العزلات وتم تقدير إنتاج Siderophores بواسطة عدة طرق منها CAS agar والطرق اللونية. تم الكشف عن اثنين من الجينات بواسطة تفاعل البوليميراز المتسلسل (PCR). كما تم دراسة العلاقة بين هذه الجينات واللزوجة العالية من جهة، ومن جهة أخرى تم دراسة العلاقة بين هذه الجينات وإنتاج Siderophores.

أظهرت النتائج أن ٥١٪ من العزلات كانت موجبة لاختبار String و ٤٩٪ كانت سلبية لهذا الاختبار. وأظهرت النتائج أن إنتاج Siderophores تراوح ما بين (74 - 14) psu. كما أظهرت نتائج الدراسة الجزيئية أن ٢٣٪ من العزلات احتوت على جين *iucA* و ١٧٪ احتوت على *iroB*. بينت النتائج وجود ارتباط كبير بين الجينين وإنتاج الغشاء المخاطي. وكذلك فإن إنتاج Siderophores كان مرتبطاً بشكل كبير بامتلاك أحد الجينات أو كليهما بأكثر من (60 psu) من إنتاج عوامل الحديد.

الكلمات المفتاحية: عالية الضراوة، الكليسيلا، الجزيئي، المظهري، السايديروفور.