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Molecular Study of Siderophore Genes in Carbapenems Resistant *Klebsiella pneumonia*e Isolated from Urinary Tract Infection Patients

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Abstract: Urinary tract infections (UTIs) frequently result from gram-negative, bacillus bacteria one of them is called *Klebsiella pneumoniae* .These pathogens are capable of acquiring genes that code for a variety of antimicrobial resistance mechanisms, such as carbapenem resistance. The goal of the presented work was to identify siderophore genes in carbapenemase *K. pneumoniae* bacterial isolates from urine samples collected from patients with complicated UTIs at five hospitals in Baghdad, Iraq. From November 2022 until the end of February 2023, samples have been collected,from five hospitals in Baghdad.A total of 180 urine samples from outpatients and inpatients who attended were taken In contrast to the ratio of male patients (38.33%), the majority of patients (61.67%) were female. In sterile containers, midstream urine (MSU) samples have been taken from patients exhibiting UTI symptoms. The samples have been incubated at of 37° C for 24 hrs while being grown on MacConkey agar, blood agar, and Hi Chrome UTI Agar. Through watching grown colonies and isolating Gram-negative bacteria, the outcomes were documented. Out of (180) clinical samples ,n=50 (27.77 %) isolates were recognized as *K.pneumoniae* ,then selected (10) resistance isolates to made the detdction of *entB* and *ybtS* genes .In this study, all n=10 (100%) isolates were determined positive for *16srRNA* housekeeping gene, n = 10 (100%) of isolates were determined positive for the *ybtS* gene, and n = 9 (90%) of isolates were positive for *entB* gene. The current study was concluded, the high virulence factor regarding carbapenems CRKP could be due to large part to high rate of the detection of *ybtS* and *entB* genes in highly virulent CRKP.

Keywords: Carbapenems resistance, *Klebsiella pneumonia, ybtS, entB*.

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Introduction

A gram-negative bacillus known as *K. pneumoniae* could subsist as a commensal bacterium in nasopharyngeal as well as intestinal tracts (1). Because of its hypervirulence and antibiotic resistance, *K. pneumoniae* is presently one of the life-threatening bacterial pathogens and is regarded as a "critical priority" for research and the creation of new antibiotics. There are few effective treatments for the increasing frequency of severe infections caused by *K. pneumoniae* (2). *K. pneumoniae* is an enterobacterales family member and one of the opportunistic pathogens which majorly affects immunocompromised or hospitalized individuals. *K. pneumoniae* is responsible for a wide variety of ailments, including bacteremia, sepsis, pneumonia, and UTIs. The increasing prevalence of clinical bacterial isolates that are resistant to antibiotics is one of the largest stressors on healthcare systems worldwide. In order to curb the spread of MDR bacteria, it is crucial to comprehend the genetic components of antibiotic resistance (3). Hypervirulent *K. pneumoniae* (hvKP) and classical *K. pneumoniae* (cKP) are the two kinds of *K. pneumoniae* that have emerged. With regard to immunocompromised hosts,

the opportunistic pathogen cKP is primarily linked to nosocomial infections (4). A few of the virulence factors present in *K. pneumoniae* include lipopolysaccharid (LPS), siderophores, capsularpolysaccharide (CPS), and adhesion factors. All of such factors contribute to the bacteria's ability to spread to different sections of the body and inevading the host's immune system (5). Siderophore are iron-chelating compounds with a low molecular weight (less than 10 KD) that are synthesized by several bacteria. Siderophore forms complexes with free iron in huge quantities under iron limited conditions and transports them into cell with the use of the membrane receptor molecules, which are encoded through 5 genes in an operon, which is turned off once the cell has taken up enough iron. (6). *K. pneumoniae, E. coli, S. aureus,* and *S. pneumoniae* are common with antibiotic-resistant isolates (7). Asymptomatic *K. pneumoniae* carriers serve as reservoirs, aiding in the nosocomial spread regarding such bacteria between patients. *K. pneumoniae* represents a serious concern to the public health since the multidrug resistance makes it possible for such organisms to stay in the hospital environment and in the gastrointestinal tracts of both patients and personnel, as well as drastically reducing the therapeutic alternatives (8). A large spread of diseases brought on by multidrug resistant bacteria is accompanied by population growth. The most prevalent hospital-and community-acquired bacterial infection is UTI, which is frequently accompanied by high treatment failure rate and recurrence of infection. Most bacterial infections are characterized by a high treatment failure likelihood and infection recurrence (9). Meropenem, ertapenem, and imipenem are examples of carbapenem antibiotics that are regarded as having the highest therapeutic value in hospitals. In situations of severe infections brought on by bacteria that produce the Extended Spectrum β-lactamase (ESBL) enzymes, antibiotics are advised as the first treatment line as a result of their broad spectrum of activity. As such enzymes are not just the carbapenems, yet also cephalosporins, monobactams, and penicillins, the advent of the carba penemase is becoming a growing therapeutic issue. Resistance to numerous antibiotics, including β-lactam and non β-lactam antibiotics, is typically connected with resistance to carbapenems antibiotics. Because these infections have been caused by carbapenemase-producing *K. pneumoniae* organisms, which are difficult to treat because of their high resistance, fatality rates for those infections increased (10).

The present study was aimed to determine whether the *ybtS* and *entB* genes are present in extremely virulent carbapenems *K.pneumoniae*.

Materials and methods

Collection of samples

At the Al Kindi Teaching and Hospital Medical City Baghdad, Iraq, 180 midstream urine samples (MSU) from patients with UTI of both sexes and all ages were obtained (Ghazi Al-Hariri Hospital for Surgical Specialties, Baghdad Teaching Hospital, Paediatric Teaching Hospital and Teaching Laboratories). Identification of *K. pneumoniae* manual biochemical tests that were utilized for final embedding in VITEK2 compact system from the beginning of November 2022 to the end of February 2023.

Antimicrobial susceptibility testing

In accordance with Coudron *et al*., this test was performed (11). A 0.5

McFarland standard was established for each isolate. In order to test for antibiotic sensitivity, the standard suspension has been spread over Mueller-Hinton agar plates and allowed to dry for five minutes. The *K. penumoniae* isolates (50) have been tested against 12 antibiotics Ceftazidime (CAZ), Ceftriaxone (CTX), Nitrofurantoin (F), Trimethoprim (TMP), Gentamicin (GM), Nalidixic acid (NA), Imipenem (IPM), Amoxicillin Clavulanate (AMC), Tobramycin (TOB), Meropenem (MEM) and Ciprofloxacin (CIP) and Chloramphenicol (C) with the use of disc diffusion method. The plates were then turned over and incubated for 24hrs at 37°C. In accordance with the Clinical and Laboratory Standards Institute, the inhibitory zones that the discs created were measured with the use of a metric ruler in millimeters (mm) (12). The isolates were categorized as intermediate, susceptible, or resistant to the antibiotic by comparing them to usual inhibitory zones.

Extraction and measured concentration and purity of DNA

Bacterial DNA extraction

The DNA has been extracted from *K.pneumoinae* isolates that have been diagnosed by the Vitek-2 system using the extraction kit processed by NEB® (England) the extraction results were going without problems.

Identification of *K. Pneumoniae* and carbapenemase

DNA extraction

Through employing the specific primers in table (1), the genes encoding the siderophore genes resistanc determinants, *entB* and *ybtS,* were examined. Each primer underwent a five-minute step of initial denaturation at 94^{o}C, followed by 30 cycles of 94^{o}C for 30sec, 50^{o}C for 45sec, and 72^{o}C for seven minutes, in a 25 µL PCR amplification process, as shown in Table (2).

Table (1): Sequences of the primers that have been utilized for Conventional PCR to Detect *K. pneumoniae ybtS, entB* and *16srRNA* Genes.

Gene	Oligonucleotide primer sequence 5' to 3'	PCR product size (bp)	Ref.
ybtS	**F**: GACGGAAACAGCACGGTAAA	**242**	**(13)**
	R: GAGCATAATAAGGCGAAAGA		
entB	**F**: GTCAACTGGGCCTTTGAGCCGTC	**400**	**(13)**
	R: TATGGGCGTAAACGCCGGTGAT		
16s rRNA	**F**:: CAGCTCGTGTCGTGAGATGT	**150**	**(14)**
	R: CGTAAGGGCCATGATGACTT		

Table (2): Component of PCR Master Mix Reaction. The cycling conditions of the 16srRNA gene of *K. pneumoniae* (15)

PCR Master mix reaction components		Volume
Master Mix or GoTaq ® Green Master Mix		12.5µl
Template of the DNA		1.5µl
Primers	**Forward**	1µL
	Reverse	1µL
nuclease-free H_2O		9µL
Total volume		25Ml

Polymerase chain reaction (PCR)

2.5µl of the DNA template (60 ng/µl) were amplified in a 25 µL PCR reaction with the use of 12.5µl of G2 Go Taq® green master mix (Promega/US) and 0.5µl of every primer (10 pmol/µl)

for the gene, up to final volume of 25µl with nuclease-free water. The PCR premix, primers, and extracted DNA were defrosted at 4°C, vortexed, then briefly centrifuged to force the contents to the tubes' bottoms. After a number of tests, the PCR was optimized. the negative control was contained all material but without DNA.Therefore, nuclease-free water was added rather than run of template DNA. Programs PCR were set on the Thermal-cycler (Applied Biosystems, USA), PCR Thermocycler quantities were performed by using the Conventional PCR Thermocycler, that described in Table (3) and (4).

Table (3): pcr thermoCycling Conditions for 16S rRNA Genes (16) (17).

Loop's steps	Temp. (ºC)	Time	Number of cycles
Initial denaturation	95	5min	1
Denaturation	95	45sec	30
Annealing	60	45sec	
Extension	72	45sec	30
Final extension	72	5min	1

Table (4): PCR Thermo-Cycling Conditions for *ybtS* and *entB* Genes (16) (17)

Loop's steps	Temp.(ºC)	Time	Number of cycles
Initial denaturation	95	5min	1
Denaturation	95	45sec	30
Annealing	60	45sec	30
Extension	72	45sec	
Final extension	72	5min	1

Results and discussion

Isolation and identification of *K. pneumoniae*

According to Parven *et al* (18). Following morphology identification by Gram's staining, cultural properties, and biochemical characteristics, bacteria were isolated and identified. The culture media used for it are as follows: Streaking all the samples (clinical and hospital samples) on MacConkey agar, Blood agar, and HI chrome UTI agar, and incubated at a temperature of 37^{o}C overnight for 24 hrs. The following characteristics of isolates grown from these media were used to identify them. The positive result for *K.pneumoniae* on MacConkey agar is typically displayed on formed bright pink colonies with mucoid structure due to fermenters of the lactose sugar in the media, which is a feature of this bacteria. While blood ager appeared as non-hemolytic grey white colonies, cultivated on the blood agar, they did not produce blood hemolysis (19).

HI chrome UTI agar *Klebsiella*, a highly selective and sensitive media for *K.pneumoniae* (appeared as metallic blue. About 50 isolates of *K.pneumoniae* were confirmed on the HI Chrome UTI agar, which is a diagnostic and differential media between the isolated through my employment for diagnostic bacteria from UTI samples that were grown on the conventional culture media that were previously identified and then diagnosed these isolates.

Distribution of bacterial isolates isolated from UTI.

The VITEK 2 system on the samples of the current study all (180) samples were collected from Midstream urine MSU samples in sterile containers from patient with infection of UTI, isolated from urine samples The distribution of pathogenic investigation is shown in Table (5).

Table (5): Numbers and percentage of the total specimens (N=180).

Bacteria	No.	Percentage %
Klebsiella pneumonia	50	27.8 %
E.coli	54	30%
Pseudomonas aeruginosa	8	4.44%
Proteus mirabilis	6	3.33%
Staphylococcus aureus	11	6.11%
Enterococcus spp	7	3.9 %
Acinetobacter baumannii	4	2.22%
Candida albicans	4	2.22%
No growth	36	20%
Total	**180**	**100**%

Table (5) shows that; One hundred and eighty urine samples (MSU) have been collected from patients with UTI of all ages and both sexes. Samples were collected from 180 urine samples; preliminary results showed growth in 144 (80%) specimens and 36 samples (20%) without growth, *E. coli* had the highest percentage, representing (30%), followed by *Klebsiella pneumonia* with (27%). Followed by *Pseudomonas aeruginosa* (4.44%), and *Proteus mirabilis* (3.33%), *Staphylococcus aureus* (6.1%) *Enterococcus* spp (3.9%) *Acinetobacter baumannii* (2,22%) and *Candida albicans* (2.22%). This study agrees with Hasan *et al*. (20), in Iraq by found that *E. coli* was the most frequent cause of UTIs in the patients (30.38%), led *by Klebsiella pneumonia* (26.14%).

As for age, the age groups of(21-30) years had the highest rate of infection with 22.8%, Followed by (11-20) years with a rate of 21.1%,The group (1-10) were 11.7%, while the group (31-40) were rate 16.1%, and (41-50) were rate 17.8%, The lowest group (61-80) were rate 2.7%, and (51-60) were 7.8%. It was found that the rate of infection in females was 61.67% and in male 38.4% which represent in table (6).This study similar to Khalid, (21) study rate was females (65.7%)males (34.3%).

Table (6): Distribution of specimen collection from patients with UTI according to age and gender.

Age	Male	%	Female	%	Total	%
1- 10 years	12	6.7%	9	5%	21	11.7
11 - 20 years	16	8.9%	22	12.2%	38	21.1
21 - 30 years	13	7.2%	28	15.6%	41	22.8
31 - 40 years	11	6.1%	18	10%	29	16.1
41 - 50 years	10	5.6%	22	12.2%	32	17.8
51 - 60 years	5	2.8%	9	5%	14	7.8
61 - 80 years	2	1.1%	3	1.6%	5	2.7
Total	**N=69 (38.33 %)**	**38.4%**	**N=111 (61.67 %)**	**61.6%**	**180**	**100%**

According to the table (7), the antibiotic susceptibility test was carried out for *k.pneumoniae* by Kirby Bauer disk diffusion method on a Mueller-Hinton agar according to all identified *K. pneumoniae* were exposed to antimicrobial agents .The results showed all *k.pneumoniae* had been resistant to Trimethoprim (100%) and highest resistance to Amoxicillin/ Clavulanic

acid at (92%) Ceftazidime (72%), and Ceftriaxone (70%) also moderate resistance has been noticed for antibiotics Chloramphenicol (60%) and Ciprofloxin (60%), Nitrofurantoin (46%), Gentamycin (42 %) Moderate resistance was observed for Nalidixic acid (36%), and Tobramycin (36%), And the Lowest resistance was Meropenem (20%), Imipenem (14%). In local study by Rhumaid (22) referred is susceptibility of isolates were Imipenem (5.60%), Meropenem (9.40%), Ceftazidime (58.50%), Ceftraixone (50.90%) and Cefotaxime (43.30%).

And another study done by Hussain (23), reported that the highest resistance percentage was towards Trimethoprim\ Sulfamethoxazole 85.71% followed by Ceftriaxone 83.92%, Gentamycin 66.07%, Amoxicillin/ Clavulanic acid 42.85%, Pipracillin 26.79%, Ciproflaxacin 25% and Impeneme 17.86% for 56 *K. pneumoniae* isolates.

Table (7): Antimicrobial susceptibility test of 50 *K. pneumonia* isolates against 12 antimicrobial agents.

Antibiotics		Disc content(μ g)	No. isolated	R	No. isolated	I	No. isolated	S
Meropenem	MEM	10	10	20%	2	4%	38	76%
Imipenem	IPM	10	7	14%	3	6%	40	80%
Amoxicillin/ Clavulanic	AMC	10	46	92%	0	0%	4	8%
Ceftazidime	CAZ	30	36	72%	0	0%	14	28%
Ciprofloxin	CIP	5	30	60%	0	0%	20	40%
Chloramphenicol	C	30	30	60%	5	10%	15	30%
Ceftraixone	CTX	30	35	70%	5	10%	10	20%
Nitrofurantoin	F	100	23	46%	17	34%	10	20%
Gentamycin	GM	10	21	42%	0	0%	29	58%
Nalidixic acid	NA	30	18	36%	6	12%	26	52%
Trimethoprim	TMP	10	50	100%	0	0%	0	0%
Tobramycin	TOB	10	18	36%	5	10%	27	54%

In another study reported by Mohammad (24), revealed that the high antibiotic resistance level has been against Amoxicillin/ Clavulanic acid and Ceftriaxone 80.95%. In other study, it has been observed that *K. pneumoniae* showed lowresistance to older drugs like chloramphenicol by Kumar (25).

Molecular identification of *K. pneumonia* by detection of *16SrRNA* gene

The study looked at a total of n=10 carbapenem-resistant isolates. These isolates were found in clinical urine samples. Identification using the specific initiator of the *16S rRNA* gene to support the diagnosis made by PCR. The findings revealed that all bacterial isolates belonged to the species *K. pneumoniae.*

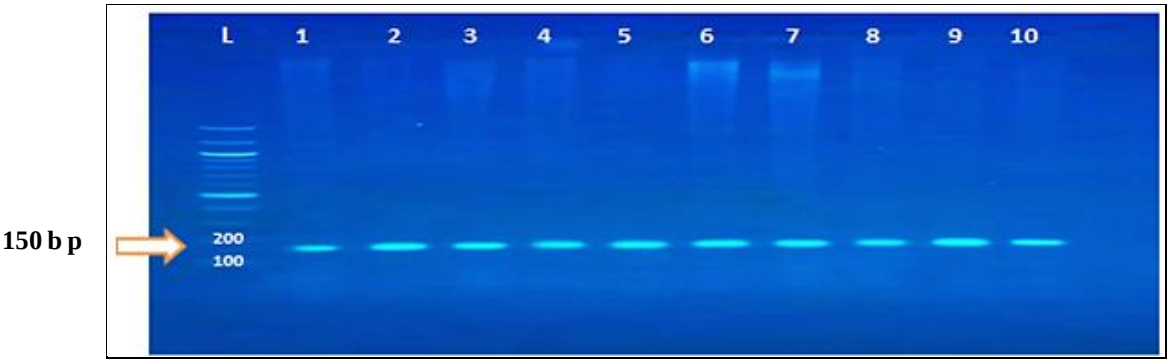

Figure (1): The gel electrophoresis of the PCR product of 16SrRNA gene (150 bp) of *K.pneumoniae* 100bp DNA ladder; (1% Agarose, 90 V for 70 mint).

The positive isolates identified by Vitek 2 system assay gave positive results in the detection of *16SrRNA* gene by polymerase chain reaction amplification. The PCR products were confirmed by the analysis of bands with gel electrophoresis and by comparison of bands molecular size with DNA Ladder of 100 bp. The results of reaction for *16S rRNA* gene shown in PCR Figure (1).

Detection of *K. pneumoniae ybtS and entB* genes

In order to detect the presence of *K.pneumoniae ybtS* and *entB* genes. The PCR products were confirmed by the analysis of bands with gel electrophoresis and by comparison of bands molecular size with DNA Ladder of 100 bp. The positive result isolates are n=10 (100 %) isolates were positive for the *ybtS* gene shown in figure (2). And n= 9 (90%) isolates were positive for *entB*, shown in figure (3); And this agree to Aljanaby (26), in Iraq reported that the most commen virulence genes were *entB* (100%).And in other study showed present the *entB*, (99.6%) by Hu *et al.*, (27), In a different study that has been reported by Zhan (28). from China found the virulence-associated genes among 21 isolates included *entB* 95.2%, *ybtS* 95.2% . Another study by Han. (29), showed that the strong virulence strains were carrying *entB* as well as *ybtS* genes.

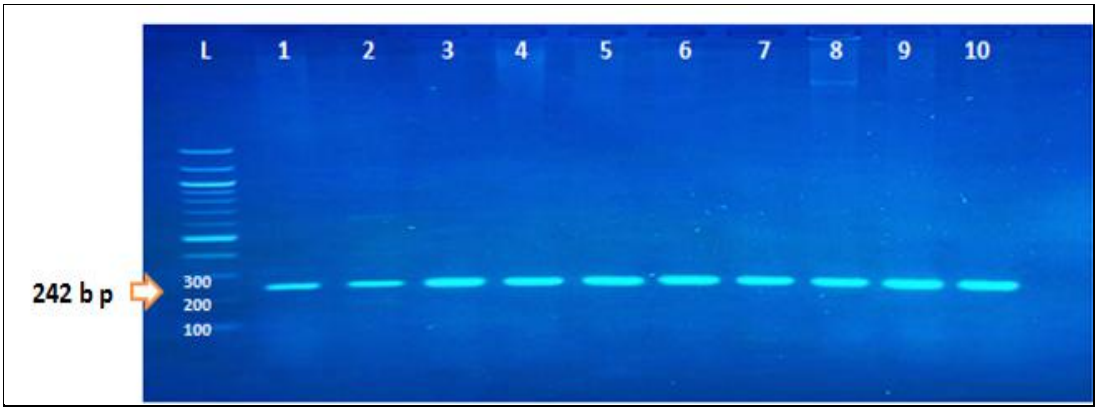

Figure (2): The gel electrophoresis of PCR product of the *ybtS* gene (242bp) of *K.pneumoniae* 100bp DNA ladder; (1% Agarose, 90 V for 70 mint)

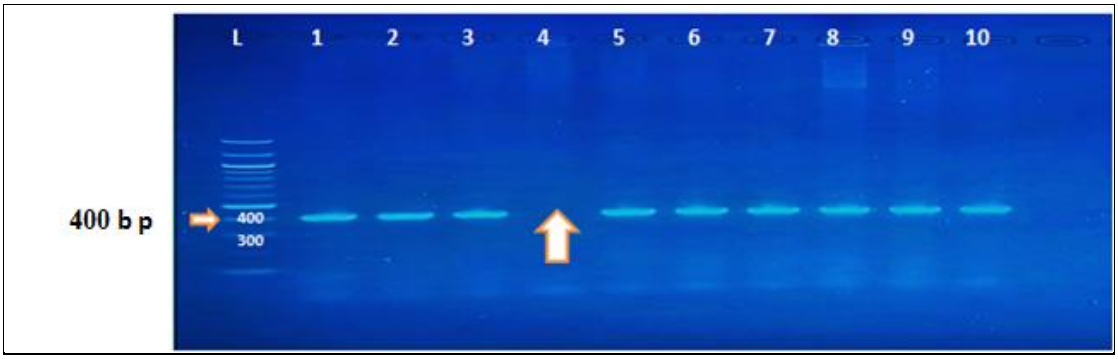

Figure (3): The gel electrophoresis of the PCR product of *entB* gene (400 bp) of *K.pneumoniae*, 100bp DNA ladder; (1% Agarose, 90 V for 70 mints)

The CRKP strains with *entB* and *ybtS* genes could significantly increase siderophore production. Although both the *entB* and *ybtS* genes could impair the growth rate of the of CRKP strains. In previous study,was proved the presence of *entB* and *ybtS* genes reduced the survival rate of mice infected with CRKP strains.

CRKP strain, the role of *ybtS* gene was relatively slow. *entB* and *ybtS* genes enhanced the antiserum killing abilityHistopathological changes and and livers of infected mice were enhanced by the presence of *entB* and *ybtS* genes. Mice infected with the same strain had higher histopatho logical changes and levels of inflammatory factors in the lungs than in the livers.The siderophore virulence genes *entB* and *ybtS* have no significant effect on the colony morphology, mucus phenotype and biofilm formation ability of CRKP strains.And the siderophore virulence genes *entB* and *ybtS* can significantly enhance the virulence of the CRKP strain(29).

Conclusion

This study revealed that *k.pneumoniae* bacterial uropathogens were widely distributed and many isolates had a high rate of resistance to the carbapenems, one of the last resort classes of antibiotics, in two significant tertiary care institutions in Baghdad, Iraq. And demonstrated that the key siderophore virulence genes linked to high rate of detection in high siderophore production carbapenemase resistance *K. pneumoniae* are *entB* and *ybtS*.

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