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# Determination of some virulence factors of *Citrobacter freundii* isolated from Iraqi patients

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**Abstract**

This study included the isolation and identification of *Citrobacter freundii* from 220 samples collected from inpatients and outpatients suffering from urinary tract infection (UTI) and identified at the laboratory of the General Samarra Hospital in Samarra City, Iraq. The study was conducted to investigate some of the virulence factors produced by *C. freundii*. The results showed that 67 isolates were belonging to the *C. freundii*, with a rate of 30.45%. Twenty eight samples were from inpatients (41.8%) and 39 samples were from outpatients. The bacterial identification was based on cultural and biochemical tests and confirmed by using VITEK2 compact system. Virulence factor results showed that all isolates were not blood hydrolyzing whereas they produced protease. Seven isolates (10.4%) produced biofilm, five from inpatients and two from outpatients, at rates of 17.8% and 5.1%, respectively. The results showed that 17 (25.4%) of the pathogenic isolates were β-lactamase producers, as determined by the iodometric method, twelve of them (17.9%) were from inpatients and 5 (7.5%) from outpatients. Four isolates of *C. freundii* produced Extended Spectrum Beta-lactamase (ESβL) enzymes, three from inpatients and one from outpatients, with ratios of 4.5% and 1.4%, respectively. Also, the *via B* gene, which is responsible for virulence factors, was investigated using PCR. The results showed that 12 isolates from inpatients and 4 isolates from outpatients were harboring this gene. The antimicrobial susceptibility testing by Kirby-Bauer's method showed that all isolates that produced β-lactamase were resistant to antibiotics.

**Key words:** *C. freundii*, virulence factors, Biofilm, β-lactamase, ESβL, PCR.

# تحديد بعض عوامل ضراوة بكتريا *Citrobacter freundii* المعزولة من المرضى الراقدين والوافدين لمستشفى سامراء العام

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**الخلاصة**

تضمنت الدراسة عزل وتشخيص بكتريا *Citrobacter freundii* من 220 عينة جمعت من المرضى الراقدين والوافدين المصابين بالتهاب المسالك البولية بعد ان حددت الحالات في مختبر المستشفى في مستشفى سامراء العام في مدينة سامراء. أجريت هذه الدراسة للتحري عن بعض عوامل ضراوة بكتريا *C. freundii*. بينت النتائج ان 67 عزلة تعود لبكتريا *C. freundii* وبنسبة 30.45% .ثمانية وعشرون عزلة من المرضى الراقدين وبنسبة 41.8% و 39 عزلة من المرضى الوافدين . اعتمد التشخيص على الاختبارات الزرعية

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والكيموحيوية، وتم تأكيد التشخيص باستخدام جهاز الفايتك2. اظهرت نتائج عوامل الضراوة ان جميع العزلات كانت غير محللة للدم بينما كانت منتجة لأنزيم البروتييز. سبع عزلات (10.4%) كانت منتجة للغشاء الحيوي biofilm، خمسة منها من المرضى الراقدين وعزلتان من المرضى الوافدين وبنسبة 17.8% و 5.1 % على التوالي. كما بينت النتائج ان (17) 25.4% من العزلات الممرضة كانت منتجة لإنزيمات البيتالاكتاميز باستخدام طريقة اليود القياسية. اثنا عشرة عزلة (17.9%) من المرضى الراقدين و 5 عزلات وبنسبة 7.5% من المرضى الوافدين. اربع عزلات من بكتريا *C. freundii* كانت منتجة لأنزيمات البيتالاكتاميز واسعة الطيف ESβL ثلاثة منها من الراقدين وعزلة واحدة من الوافدين وبنسبة 4.5%, 1.4% على التوالي. كما تم التحري عن جين *via B* المسؤول عن عوامل الضراوة باستخدام تقنية التضاعف التسلسلي PCR. وقد بينت النتائج ان 12 عزلة من المرضى الراقدين واربع عزلات من الوافدين كانت تمتلك هذا الجين. اظهر اختبار فحص الحساسية للمضادات الحيوية باستخدام طريقة Kirby–Bauer's method ان جميع العزلات المنتجة لإنزيمات البيتالاكتاميز كانت مقاومة للمضادات الحيوية.

## Introduction

*Citrobacter freundii* is a Gram negative, cocobacilli, lactose fermenter, facultative anaerobic member of the enterobacteriaceae family, which may be found in water, soil and in the gastrointestinal tracts of humans and animals. *C. freundii* causes urinary tract infections, wound infections, severe diarrhea, pneumonia, neonatal meningitis and brain abscesses in humans, especially in immunocompromised hosts [1]. *C. freundii* is considered as a major cause of nosocomial infections, characterized by inducible resistance mediated by the chromosomal AmpC β –lactamase [2]. Overtime, *C. freundii* isolated from hospitals patients suffering from urinary tract infection becomes more resistant to different antibiotics like beta-lactamase, third generation cephalosporins and aminoglycosides [3]. *C. freundii* has several virulence factors which are considered as means used by pathogenic microorganisms to express their pathogenicity and promote their survival in the hosts. Those virulence factors are closely related to the disease and can be elicited during antimicrobial therapy [4]. Virulence factors, including proteolysis, hemolysis and biofilm formation, have also been previously observed [5]. For example, diarrheagenic biofilm formation was discovered in *C. freundii* [6]. The diseases caused by *C. freundii* can be either community acquired or nosocomial infections [7]. Studies of *C. freundii* demonstrated their potential risks due to the production of some virulence factors which contain the genes of resistance and pathogenicity that convert these bacteria into a more deadly and resistant organism [8]. Therefore, the present study aimed to investigate the different percentages of the virulence factors of *C. freundii* isolated from inpatients and outpatients suffering from UTI.

## Materials and methods

### Isolation and diagnosis of bacterial isolates

All pathogenic bacteria were obtained from the inpatients and outpatients in the General Samarra Hospital. The bacterial isolates were cultivated on selective and differential media (MacConkey agar, Blood agar, Mannitol salt agar) in the laboratory, where they were stained by Gram stain and subjected to some biochemical tests [9]. To confirm the identification of bacterial isolates, VITEK2 compact system was performed. All the collected isolates were isolated from urinary tract infections.

### Virulence factor tests

### Hemolytic activity

Plate method was used to detect the hemolysis activity . Each isolate was streaked onto 5% sheep blood agar (Himedia, India) and incubated at 37° C for 24 hours. Isolates exhibiting hemolytic zones surrounding the growth were considered positive [10].

### Proteolysis test

To detect the proteolysis of bacterial isolates, bacterial suspension was prepared by culturing a single colony of each isolate in 5 ml nutrient broth and incubated at 37°C for 18 hours. 100 µl of each suspension was transported to the prepared wells in skimmed milk agar plates. The plates were incubated at 37°C for 24-48 hours. The diameter of lysis was measured [11].

### Biofilm production

Formation of biofilm was tested using Congo red agar (CRA) plates. CRA plates were inoculated with tested bacteria and incubated at 37$^{o}$ C for 24 to 48 hours aerobically. Black colonies with a dry

crystalline consistency indicated biofilm production [12].

**β-lactamase test**

The standard iodine method was used to detect the β-lactamase production. Overnight bacterial cultures were prepared and the bacterial colonies were transferred to 100 µl of penicillin G solution, and the tubes were incubated for 30 minutes at 37 ° C. Fifty µl of starch solution were added to each tube and mixed well. To each tube, 20 µl of iodine solution were added. The result was considered as positive upon the rapid color change from blue to white, i.e. within 5 minutes after adding the iodine reagent [13].

**Extended spectrum β-lactamase test**

All isolates were examined by disc approximation tests for identifying the ability of bacterial isolates to produce ESβL. Increasing the inhibition zone between the disc of Augmentin and the discs of Piperacillin, cefotaxime, and Ceftriaxone indicates positive reactions [14].

**Polymerase Chain Reaction**

PCR technique is used to investigate the genes responsible for bacterial virulence factors**.** The primer pair VIAB-1 from 5867 bp to 5888 bp (TGTCGAGCAGATGGATGAGCAT) and VIAB-2 from 6362 bp to 6383 bp (ACGGCTGAAGGT TACGGACCGA) was used. This primer pair will amplify and produce a double-stranded fragment of 515 bp. The primers were prepared according to the instructions of the manufacturer company (Microgen company). The mixture of PCR was set up in 25µl as a final volume that contains 5 µl of PCR green master mix (1X/ µl) and 1 µl (10 pmol) of each of forward and reverse primers. DNA template (1.5 µl in concentration of 50ng/µl) and distilled water (16.5 µl) were added to form the final volume. The tubes containing the samples were placed in thermocycler device to perform PCR according to the appropriate amplification program for each primer, as described previously [15].

**Antibiotic Susceptibility Test**

The disc diffusion method was used to detect antibiotic sensitivity of the isolates using the Kirby-Bauer method cited by an earlier work [16]. The results were compared with the standard diameter of inhibition zones for each antibiotic [17]. Eighteen antibiotic discs were used in this research (Bioanalyse/ India).

**Results and Discussion**

**Isolation of bacterial samples**

Two hundred twenty samples were collected from the inpatients and outpatients suffering from UTIs. The results showed that 67 samples belonged to *C. freundii*, 28 (41.8%) from inpatients and 39 (58.2%) from outpatients, as shown in Table-1. The results of this study are inconsistent with the findings of a previous work [18].

**Table 1-**Bacterial numeration rates of *C. freundii*

| Groups | Samples | | *C. freundii* | |
|---|---|---|---|---|
| | No. | % | No. | % |
| Inpatients | 70 | 31.81 | 28 | 41.8% |
| Outpatients | 150 | 68.19 | 39 | 58.2% |
| Total | 220 | 100 | 67 | 100% |

**Identification of bacterial isolates**

The results in Table-2 show the outcomes of the cultural and biochemical tests that were performed on the isolates under study. These results are consistent with those of the approved diagnostic systems [9-11].

**Table 2**-Biochemical and morphological properties results of *C. freundii*

| lactose fermentation | coagulase | IMVIC | | | | Motility | Hemolysis | Urease | oxidase | catalase | Gram stain | *C. freundii* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C | VP | MR | IND | | | | | | | |
| + | - | + | - | + | - | + | - | V | - | + | $G^{-ve}$ | Tests |

G-ve: Gram negative bacteria, IND: indole, MR: methyl red, VP: Voges proskaur, C: Citrate utilization, V: variable

**Virulence factor tests**

The results of the hemolysis pattern showed no hemolysin activity on sheep blood agar plates of all the bacterial isolates under study. The results are consistent with those found a previous study [19].

In contrast, all isolates showed the ability to produce protease enzyme on skimmed milk agar. This enzyme is one of the important virulence factors that hydrolyzes peptide bonds and thus helps bacteria to invade another tissue [20]. The results disagree with those reported earlier [19], which found that only 36% of *C. freundii* were protease producers.

Seventeen isolates were shown to produce β-lactamase, 17.9% from inpatients and 7.5% from outpatients, as listed in Table-4. The results disagree with those of other authors [21] who reported that all *Ctrobacter* species were β-lactamase producers.

The results listed in Table-3 show the ability of 4 isolates of *C. freundii*, out of 67 isolates, to produce ESβL enzyme, 3 from inpatient (4.5%) and one from outpatient. This result is similar to that of another investigation [22], which found that 2.6% of *C. freundii* were ESβL producers. Whereas another work [23] reported that 63.3% of the isolates were producers of this enzyme.

**Table 3**-Percentage of production of β-lactamase and Extended Spectrum Beta-lactamase from isolates of *C. freundii*

| Groups | No. | β-lactamase + | -β-lactamase | ESβL + | ESβL - |
|---|---|---|---|---|---|
| Inpatients | 28 | 12 (17.9%) | 16 (23.9%) | 3 (4.5%) | 25 (37.4%) |
| Outpatients | 39 | 5 (7.5%) | 34(50.7%) | 1 (1.4%) | 38 (56.7%) |
| Total | 67 | 17 (25.4%) | 50 (74.6%) | 4 (5.9%) | 63 (94.1%) |

Table-4 reveals the bacterial numeration rate of biofilm production. The results showed the ability of some *C. freundii* isolates to produce the biofilm on CRA by giving black colonies, as shown in Figure -1. The biofilm helps the bacteria to survive in extreme conditions within the host and is responsible for chronic and acute infections, including endocarditis, cystic fibrosis, otitis media, medical device-related injuries, and catheter infections [24].

Biofilm formation by nosocomial pathogenic bacteria leads to increased resistance against antibiotics by expanding mutation rates and exchanging genes responsible of bacterial resistance to antibiotics [25]. Previous studies demonstrated biofilm formation in patients suffering from bacterial infections [26].

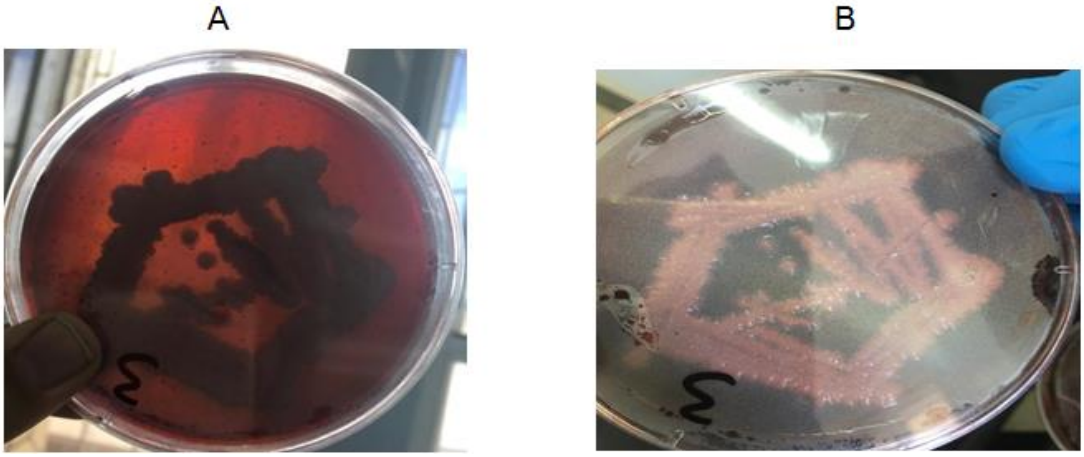


**Figure 1**-A- Positive for biofilm production, B- Negative for biofilm production

**Table 4**-Percentage of production of biofilm by *C. freundii*

| Groups | No. | biofilm + | -biofilm |
|---|---|---|---|
| Inpatients | 28 | 5 (7.5)% | 23 (34.4%) |
| Outpatients | 39 | 2 (2.9)% | 37 (55.2%) |
| Total | 67 | 7 (10.4)% | 60 (89.6%) |

**PCR Analysis of Virulence Genes**

The isolates of *C. freundii* were subjected to PCR analysis to determine the genes responsible for the production of these virulence factors. Sixty seven isolates of *C. freundii* were tested to investigate the presence of the *via* B gene**.** The results in Table-5 show the presence of *via* B gene, determined by a molecular size of 515 bp, in 12 isolates from inpatients and 4 from outpatients, as also illustrated in Figure-2. The presence of *via B* gene permits the bacteria to evade the innate immune system by expanding the host response during the infection [27]. *C. freundii* can acquire integrons, plasmids, or transposon containing resistance genes that confer this bacteria severe pathogenicity. There is also clear evidence of the presence of shiga like toxins in some strain of *C. freundii* [28]. This contrasts with a previous study which indicated that *C. freundii* isolated from animals contained no shiga like toxin genes [2].

**Table 5**-The presence of *via B* gene in the isolates under study

| Groups | No. | *via* B gene + | *via* B gene - |
|---|---|---|---|
| Inpatients | 28 | 12 (17.9%) | 16 (23.8%) |
| Outpatients | 39 | 4 (5.9%) | 35 (52.2%) |
| Total | 67 | 16 (23.8%) | 51 (76%) |

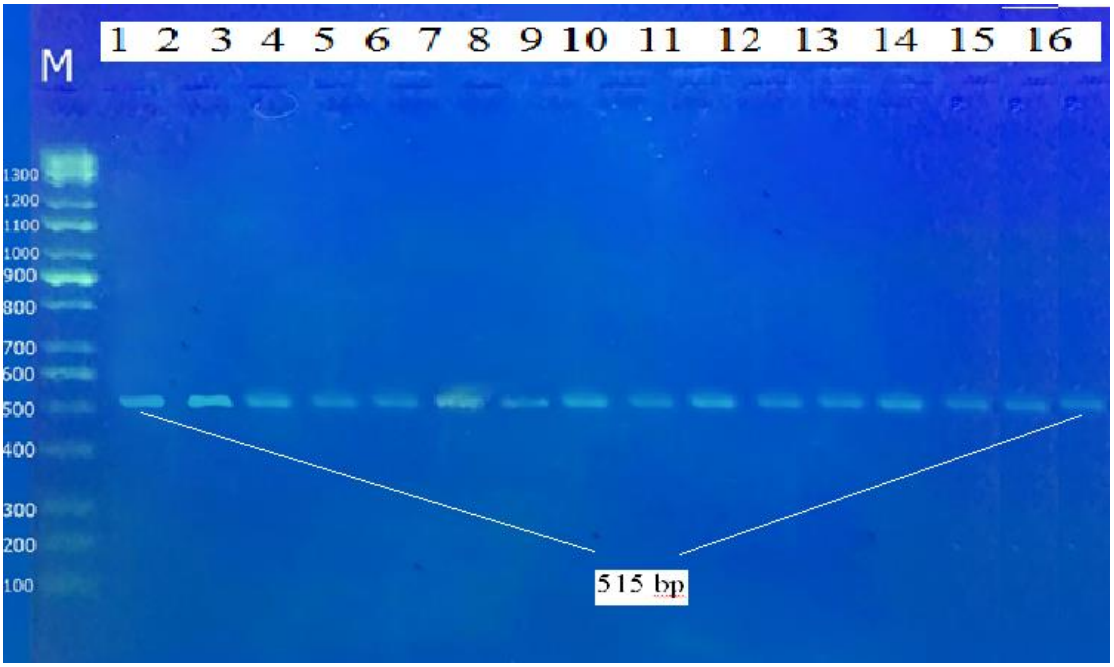


**Figure 2-** Gel electrophoresis profile of via *B* gene amplicon encoding for virulence factors by *C. freundii*. Lane M: DNA ladder 100-1500 bp. Lanes 1-12 from inpatients and 13-16 from outpatients in 515 bp represent the presence of *via B* gene on 2% agarose.

**Susceptibility of *C. freundii* to antibiotics**

The results of susceptibility test for antibiotics showed that the resistance values of *C. freundii* isolated from the inpatient to Imipenem, Augmentin and Levofloxacin were 7.1%, 17.8% and 32%, respectively. The results for the outpatient isolates were 0%, 2.5%, 46%, 12.8%, 2.5% and 25.6% resistance to Imipenem, Augmentin, ciprofloxacin, Norfloxacin, Levofloxacin and Amoxacillin, respectively. Table-6 shows that most of the isolates were highly resistant to most of the antibiotics. Also the results showed that the samples isolated from inpatients were more resistance to antibiotics compared to those from the outpatient's isolates. The factors that lead to the emergence of acquired infections might include severe illnesses, pre-hospitalization, poor nutritional status, and excessive antibiotics use. The present study showed that the majority of bacterial isolates had a relatively high resistance to β-lactam antibiotics, such as the third generation of cephalosporins. While Imipenem, a group of Carbapenems, showed a high effectiveness against all bacterial isolates. Resistance to broad-spectrum cephalosporins (third and fourth generation) is achieved through diminishing permeability through the outer membrane, higher production of AmpC β-lactamase enzymes, acquisition of

plasmids, or the combination of these factors together. The results of the present study are in accordance to those of a previous study [29], which showed the susceptibility of some Gram negative bacteria to 24 antibiotics. The authors found that most of the isolates were highly resistant to most of the antibiotics, which may be due to the production of β-lactamase. Also, the results of the present study are similar to those obtained by other authors [30], who found that most of bacterial pathogens isolated from UTI were sensitive to Imipenem.

**Table 6**-Resistance of *C. freundii* to antibiotics

| **Samples** / **Antibiotics** | Inpatients 28 | Outpatients 39 |
|---|---|---|
| Ciprofloxacin | 20 (71.4%) | 18 (46. %) |
| Piperacillin | 28 (100%) | 35 (89.7%) |
| Amikacin | 18 (64.3%) | 33 (84.6%) |
| Gentamicin | 22 (78.5%) | 28 (71.7%) |
| Imipenem | 2 (7.1%) | 0 (0%) |
| Ceftazidime | 28 (100%) | 39 (100%) |
| Cefotaxime | 28 (100%) | 39 (100%) |
| Levofloxacin | 9 (32%) | 6 (2.5%) |
| Penicillin G | 28 (100%) | 39 (100%) |
| Augmentin | 5 (17.8%) | 1 (2.5%) |
| Carbenicillin | 24 (85%) | 35 (89.7%) |
| Cefepime | 28 (100%) | 39 (100%) |
| Cephalothin | 28 (100%) | 39 (100%) |
| Ceftriaxon | 28 (100%) | 39 (100%) |
| Ampicillin | 28 (100%) | 39 (100%) |
| Norfloxacin | 23 (82%) | 5 (12.8%) |
| Cefexime | 28 (100%) | 39 (100%) |
| Amoxacillin | 22 (78.5%) | 10 (25.6%) |

## Conclusions

*C. freundii* that cause urinary tract infections have many virulence factors, as well as resistance to several antibiotics. The percentage of these factors and resistance is higher in inpatients than in outpatients, which indicates the seriousness of the nosocomial infections.

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