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Antibiotics susceptibility patterns of *Citrobacter freundii* isolated from patients with urinary tract infection in Al-Najaf governorate – Iraq

Thualfakar Hayder*[1], Ahmed Abduljabbar Jaloob Aljanaby[2]

[1]Altoosi College University, Al-Najaf Governorate, Iraq
[2]University of Kufa, Faculty of Science, Department of Biology, Iraq

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ABSTRACT

Citrobacter freundii is one of the most causative agents of urinary tract infection in human due to their high antimicrobials resistance. Therefore, this study aimed to evaluate the ability of *Citrobacter freundii* to resist to fifteen antimicrobials. A total of 461 urine samples were collected from patients infected with urinary tract infection, females and males, age groups between 18 to 60 years old performed in Al-Najaf central hospital in Al-Najaf City, Iraq, during April to December 2018. Antimicrobials susceptibility testing was performed by disc diffusion method according to the Kirby-Bauer method onto Mueller Hinton agar surface. There were 30 isolates (6.5%) diagnosed as *Citrobacter freundii*. Antimicrobial resistance rate of the 30 isolates to Amoxicillin and Chloramphenicol were all high (25 isolates 83.4%), Ciprofloxacin, Streptomycin and Sulfonamide (24 isolates 80%). The good resistance rate was observed for Penicillin, Amoxiclav and Ceftazidime (21 isolates 70%). The moderate resistance rate was observed for Cefotaxime (19 isolates 63.3%), Ceftriaxone (20 isolates 66.7%), Gentamicin (15 isolates 50%), Tobramycin (18 isolates 60%) and Erythromycin (20 isolates 66.7%). The lowest resistance rate was observed for Tetracycline (19 isolates 63.3%). While Imipenem provided full antibacterial activity (30 isolates 100%). Of the total 30 isolates, 22 (73.3%) were multi-drug resistance, 8(26.7%) extensive-drug resistance and no isolates were pan-drug resistance. While 19 isolates (63.3%) were extended-spectrum beta-lactamase producing *Citrobacter freundii*. *Citrobacter freundii* has highly resistant against most antimicrobials and became more dangerous bacteria cause urinary tract infection.

* Corresponding Author

Name: Thualfakar hayder
Phone: + 9647816118353
Email: thualfakarhh.edu11p@uokufa.edu.iq

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INTRODUCTION

Urinary tract infection is one of the most important recurrent infections infect man and women worldwide (Mohammed *et al.,* 2016; Aljanaby 2018a) mainly caused by gram-negative bacteria such as *Escherichia* coli, *Klebsiella pneumonia* and *Citrobacter freundii* (Perslev *et al.,* 2019; Aljanaby and Aljanaby 2018b). *Citrobacter freundii* (*C. freundii*) is a gram-negative bacterium, motile, oxidase negative and non-capsulated (Lakhundi *et al.,* 2017; Liu *et al.,* 2018). *Citrobacter freundii* is one of the most important gram-negative bacteria cause urinary tract infection and other diseases in human such as bacteremia (Park *et al.,* 2013; Ouyang *et al.,* 2018; Anderson *et al.,* 2018). Recently, *Citrobacter freundii* isolated from hospitals patients with urinary tract infection becomes more resistant to different antibiotics like beta-lactamase, 3rd generation cephalosporins and aminoglycosides (Aljanaby and Gafil 2013; Castanheira *et al.,* 2018; Akinbami *et al.,* 2018). In Iraq, there was no

enough studies focused on *C. freundii*, and they're resistant to different antibiotics. Therefore, the main aim of the present study is to investigate the antibiotics sensitivity pattern of *C. freundii* isolated from patients infected with urinary tract infection in Al-Najaf governorate – Iraq.

MATERIALS AND METHODS

Study design and patients

This is a cross-sectional descriptive study, performed in Al-Najaf central hospital in Al-Najaf City, Iraq, during the period from April to December 2018. A total of 461 urine samples were collected from patients infected with urinary tract infection, females and males, age groups between 18 to 60 years old.

Eligibility criteria for patients

Patients will be considered eligible for registration into this study if they fulfill all the inclusion criteria and none of the exclusion criteria as defined below;

- Patients (female or male) at least equal or more than 18 years old.
- All infected patients have been diagnosed by a physician and primary microscopic characters such as the presence of pus cells in urine samples.
- Patients should have sufficient capacity for informed consent.
- Patients should don't have any other infection.
- Patients should don't take any antibiotics for treatment.

Samples collection, culture and bacterial identification

Ten ml of mid-stream urine samples were collected in sterile disposable containers after cleaned the genitals (Aljanaby and Aljanaby 2018b). All urine samples were centrifuged at 2000 rpm for 3 min, immediately, the sediment incubated aerobically with brain heart infusion broth at 37oC for 24h, then streaked with a sterile loop on to blood agar and MacConkey agar (Oxoid™) plates. Colony Forming Units (CFUs) method was used for growing single and pure bacterial colony; all urine samples containing less than 105 CFUs/ml were excluded (Tan *et al.,* 2012). All emerged bacterial isolates were identified according to colony morphology and standard microbiological tests such as; colony morphology, gram stain, oxidase test, catalase test, imvic test, motility test, coagulase test, growth on MacConkey agar (Oxoid™) (Mac Faddin 2000).

Antimicrobials susceptibility test

Antimicrobials susceptibility testing was performed by disc diffusion method according to the Kirby-Bauer method onto Mueller Hinton agar (Oxoid™) surface (Bauer *et al.,* 1966). Fourteen different antimicrobial discs were used in this study provide from OxoidTM, the USA as follow: Ampicillin 10IU (AM), amoxicillin 25µg (AX), Amoxiclav 30µg (AMC), cefotaxime 30µg (CTX), ceftriaxone 30µg (CRO), ceftazidime 30µg (CAZ), gentamicin 10µg (GM), tobramycin 10µg (TM), ciprofloxacin 5µg (CIP), Streptomycin 10µg (S), erythromycin 30µg (E), tetracycline 30 µg (TE), Sulfonamide 300µg (SSS), Chloramphenicol (C) 30µg and imipenem 10µg (IMP). The diameters of inhibition zones (mm) were measured using a caliper measure each zone with the unaided eye and compared with clinical and laboratory standards institute (CLSI) guideline (CLSI 2017). Any bacterial isolate was resistance to at least three different antimicrobials classes considered as MDR, if any bacterial isolate was resistance to all antimicrobial classes except two or three antimicrobial classes considered as extensive-drug resistant (XDR) and when any bacterial isolate was resistance to all antimicrobials class considered as pan-drug resistant (PDR) (Aljanaby 2018c).

Statistical analysis

Fisher's exact test was used in this study for the comparison between samples by using Graph pad-prism V.10 computer software. P values less than the 0.05 level of significance were considered statistically significant (Aljanaby and Alhasnawi 2017).

RESULTS

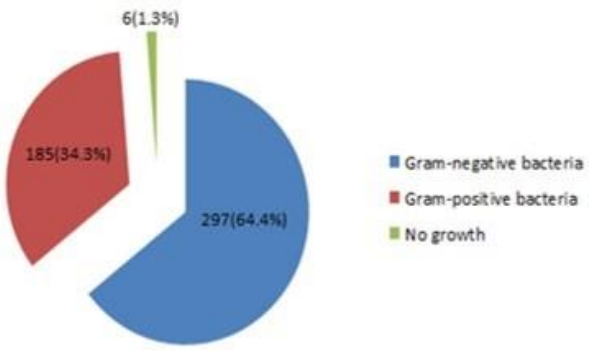

Figure 1: Numbers and percentages of total bacterial isolates from patients infected with urinary tract infection in Al-Najaf city-Iraq during the period from April to December 2018. (N=461)

Total bacterial isolates

Out of 461 different bacterial isolates from patients infected with urinary tract infection, gram-negative bacteria were the most incidences with 297 isolates (64.4%) while; there were 158 isolates (34.3%) were gram-negative bacteria and 6 isolates (1.3%) with no growth (Fig.1). According to

microscopic and culture characteristics, biochemical test, Chromagar and Vitek2@ system, out of 461 different bacterial isolates, there were 30 isolates (6.5%) diagnosed as *Citrobacter freundii*. On the other hand, out of a total of 297 gram-negative bacterial isolates, *Citrobacter freundii* was prevalent with the percentage of 10.10% (Fig.2 and 3).

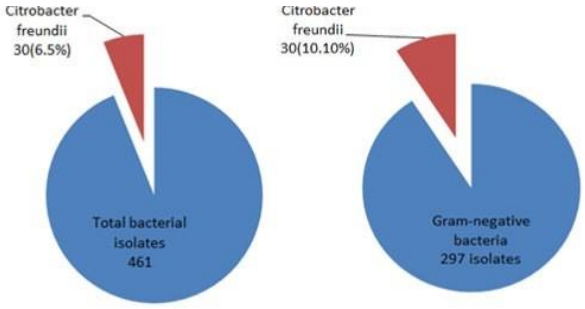

Figure 2: Numbers and percentages of Citrobacter freundii isolated from patients infected with urinary tract infection in Al-Najaf city-Iraq during the period from April to December 2018. (N=461)

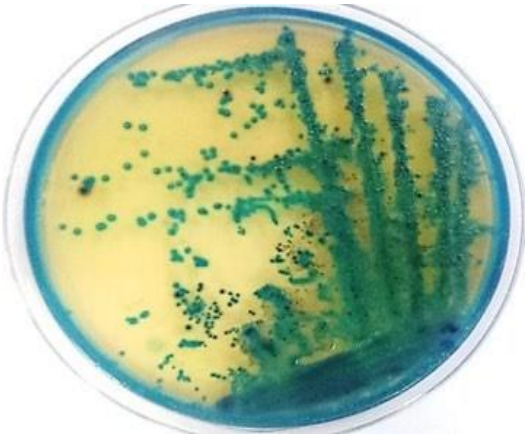

Figure 3: Citrobacter freundii grown on to CHROM agar surface after 24h of aerobic incubation at 37oC

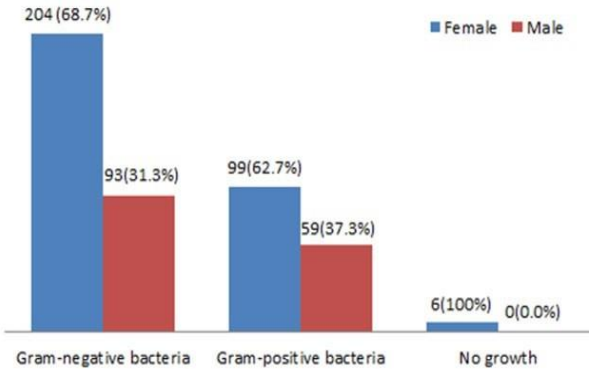

Figure 4: Numbers and percentages of total patients infected with urinary tract infection in Al-Najaf City-Iraq during the period from April to December 2018. (N=461)

Patients

The results of the present study proved that out of 461 patients infected with urinary tract infection, there were 309 patients (67%) were female, and 152 patients (33%) were male. Also, the results demonstrated that out of total 297 patients infected with urinary tract infection caused by gram-negative bacteria there were 204 patients (68.7%) were female and 93 patients (31.3%) were male. While out of a total of 158 patients infected with urinary tract infection caused by gram-positive bacteria, there were 99 patients (62.7%) were female, and 59 patients (37.3%) were male. Also, the results indicated that there were 6 patients (100%) were female infected with urinary tract infection with no bacterial growth (Fig.4). According to age groups, the results demonstrated that the age group 41-50 years old was the most prevalent range among patients infected with urinary tract infection caused by *Citrobacter freundii* (12 patients 40%, 8 females and 4 male) followed by age group 31-40 years old (8 patients 26.7%, 5 females and male), 51-60 years old (7 patients 23.3%, 5 females and 2 male) and 18-30 years old (3 female patients 10%) (Table 1).

Antimicrobials sensitivity testing

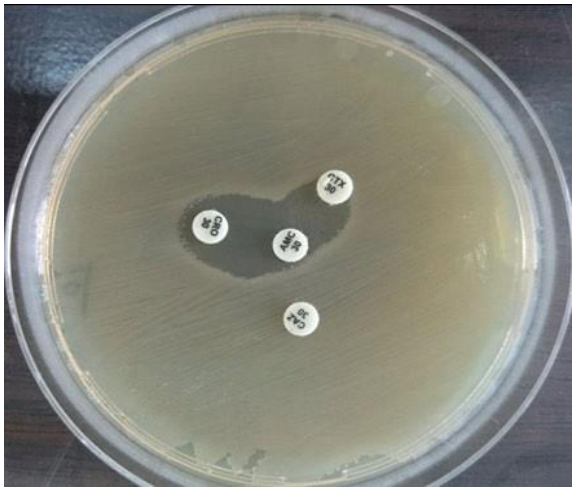

Figure 5: Modified double disc synergy test show positive result of extended-spectrum beta-lactamase producing Citrobacter freundii (isolate number 4) on to Muller-Hinton agar surface after 24h of aerobic incubation at 37°C

In this study, 15 different antimicrobials were used against *Citrobacter freundii*. The result proved that the antimicrobial resistance rate of the 30 isolates to Amoxicillin and Chloramphenicol were all high (25 isolates 83.4%), Ciprofloxacin, Streptomycin and Sulfonamide (24 isolates 80%). The good resistance rate was observed for Penicillin, Amoxiclav and Ceftazidime (21 isolates 70%). The moderate resistance rate was observed for Cefotaxime (19 isolates 63.3%), Ceftriaxone (20 isolates 66.7%), Gentamicin (15 isolates 50%), Tobramycin (18 isolates 60%) and Erythromycin (20 isolates 66.7%). The lowest resistance rate was observed for Tetracycline (19 isolates 63.3%).

Table 1: Numbers and percentages of patients infected with urinary tract infection caused by Citrobacter freundii in Al-Najaf City-Iraq during the period from April to December 2018 according to age groups. (N=30)

Age group	Female No. (%)	Male No. (%)	Total No. (%)
18-30	3	0	3 (10)
31-40	5	3	8 (26.7)
41-50	8	4	12 (40)
51-60	5	2	7 (23.3)
Total	21 (70)	9 (30)	30 (100)

Table 2: Antimicrobials sensitivity test of 30 Citrobacter freundii isolates from patients infected with urinary tract infection in Al-Najaf City-Iraq during the period from April to December 2018

Antimicrobials	Sensitive No. (%)	Resistance No. (%)
Ampicillin 10 IU	9 (30)	21 (70)
Amoxicillin 25µg	5 (16.6)	25 (83.4)
Amoxiclav 30 µg	9 (30)	21 (70)
Cefotaxime 30 µg	11 (36.7)	19 (63.3)
Ceftriaxone 30 µg	10 (33.3)	20 (66.7)
Ceftazidime 30 µg	9 (30)	21(70)
Gentamicin 10 µg	15(50)	15 (50)
Tobramycin 10 µg	12 (40)	18 (60)
Ciprofloxacin 5 µg	6 (20)	24 (80)
Streptomycin 10 µg	6 (20)	24 (80)
Erythromycin 30 µg	10 (33.3)	20 (66.7)
Tetracycline 30 µg	19 (63.3)	11 (36.7)
Sulfonamide 300 µg	6 (20)	24 (80)
Chloramphenicol 30 µg	5 (16.6)	25 (83.4)
Imipenem 10 µg	30(100)	0.(0.0)

Table 3: Numbers and percentages of multi-drug resistance and extensive-drug resistance *Citrobacter freundii* isolated from patients infected with urinary tract infection in Al-Najaf City-Iraq during the period from April to December 2018 according to age groups. (N=30)

Type of resistance	Female No. %	Male No. %	Total No. %	P value
Multi-drug resistance	16 (72.7)	6 (27.3)	22 (73.3)	0.6460
Extensive-drug resistance	5 (62.5)	3 (37.5)	8 (26.7)	Non-significant
Total	21 (70)	9 (30)	30 (100)	

Table 4: Numbers and percentages (%) of extended-spectrum beta-lactamase producing *Citrobacter freundii* isolates from patients infected with urinary tract infection in Al-Najaf City-Iraq during the period from April to December 2018. (N=30)

Extended-spectrum beta-lactamase producing *Citrobacter freundii*	Female*No. %	Male No. %	Total No. %	P-value
Extended-spectrum beta-lactamase	16 (84.2)	3 (15.8)	19 (63.3)	
Non-extended-spectrum beta-lactamase	5 (45.5)	6 (54.5)	11 (36.7)	0.0419*
Total	21 (70)	9 (30)	30 (100)	

While Imipenem provided full antibacterial activity (30 isolates 100%) (Table 2). Of the total 30 isolates, 22 isolates (73.3%) were multi-drug resistance: 16 isolates (72.7%) from female and 6 isolates (27.3%) from male, 8 isolates (26.7%) extensive-drug resistance: 5 isolates (62.5%) from female and 3 isolates (37.5%) from male and no isolates was pan-drug resistance (Table 3). According to modified double disc synergy test, the results indicated that there were 19 isolates (63.3%) were extended-spectrum beta-lactamase producing *Citrobacter freundii* 16 (84.2%) isolates from female and 3 (15.8%) isolates from male) with significant increase P= 0.0419 (Table 4) (Fig.5). The antimicrobials resistance profile of 30 *Citrobacter freundii* isolates is given in table 5.

DISCUSSION

Urinary Tract Infection is common in most region of Iraq, and it remains the main health problem in many similar developing countries (Rödel *et al.,* 2019). *Citrobacter freundii* is an opportunistic bacterium, highly adaptable and has remarkable mechanisms of survival and transmission in the host (Liu *et al.,* 2018). This pathogen is responsible

Table 5: Antimicrobials resistance profile of 30 *Citrobacter freundii* isolates from patients infected with urinary tract infection in Al-Najaf City-Iraq during the period from April to December 2018

Isolate	Sex	Age group	Phenotype resistance
1	F	51-60	AM, AX, AMC, CTX, CRO, CAZ, TM, CIP, E, TE, SSS, C
2	F	18-30	AM, CTX, CRO, CAZ, CIP, S, E, SSS, C
3	F	41-50	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, E, TE, SSS, C
4	F	31-40	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, S, E, TE, SSS, C
5	F	51-60	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, S, E, TE, SSS, C
6	F	41-50	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, S, E, TE, SSS, C
7	M	51-60	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, S, E, TE, SSS, C
8	M	41-50	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, S, E, TE, SSS, C
9	F	41-50	AM, AX, AMC, CTX, CRO, CAZ, CN, CIP, E, TE, SSS, C
10	F	41-50	AM, AX, AMC, CTX, CRO, CAZ, TM, CIP, E, TE, SSS, C
11	M	31-40	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, S, E, C
12	M	31-40	AM, AX, AMC, CTX, CRO, CAZ, TM, CIP, S, E, SSS, C
13	M	31-40	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, S, E, TE, SSS, C
14	F	41-50	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, S, E, C
15	F	18-30	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, S, E, C
16	F	18-30	AM, AX, AMC, CTX, CRO, CAZ, TM, CIP, S, E, SSS,
17	F	41-50	AM, AX, AMC, CTX, CRO, CAZ, TM, CIP, S, E, SSS, C
18	F	31-40	AM, AX, AMC, CTX, CRO, CAZ, TM, CIP, S, E, SSS
19	F	41-50	AM, AX, AMC, CTX, CRO, CAZ, CN, TM, CIP, E, TE, SSS, C
20	F	31-40	AM, AX, AMC, CRO, CAZ, CIP, S, E, SSS, C
21	F	31-40	AM, AX, AMC, CAZ, S, C
22	F	31-40	AX, AMC, S,C
23	F	51-60	AX, SSS, C
24	F	51-60	AX, S, SSS, C
25	F	41-50	AX, CN, S, SSS
26	F	41-50	S, CIP, SSS, C
27	M	41-50	TM, CIP, S, C
28	M	41-50	CN, CIP, S, SSS
29	M	51-60	CN, CIP, S, SSS
30	M	51-60	AX, AMC, SSS, C

AM: Ampicillin, AX: Amoxicillin, AMC: Amoxiclav, CTX: Cefotaxime, CRO: Ceftriaxone, CAZ: Ceftazidime, CN: Gentamicin, TM: Tobramycin, CIP: Ciprofloxacin, S: Streptomycin, E: Erythromycin, TE: Tetracycline, SSS: Sulfonamide, C: Chloramphenicol, F: Female, M: Male, No (%): Numbers and (percentages) of overall resistance, R: resistance type, MDR: Multi-drug resistance, XDR: Extensive-drug resistance, ESBL: Extended-spectrum beta lactamase producing bacteria.

for many pathogenic cases in infants and immune compromised people, also hospital-acquired infection, bloodstream, wounds infections, gastroenteritis, endocarditis, pneumonia, septicemia, urinary tract infection meningitis and brain abscesses with high mortality and morbidity (Aljanaby 2013). The antibiotics resistance is considered as a serious indicator on human health by affecting on infection severity, diseases spreading, increasing of mortality rate, and increasing of costs of antibiotics (Maraki *et al.,* 2017). Antimicrobials are the mainstay of therapy for urinary tract infection patients. However, the intensive use of first-line antimicrobials, such as ampicillin, and chloramphenicol has led to the emergence and global spread of multi-drug-resistant mainstay strains (Sirkhazi *et al.,* 2014). Infections caused by antimicrobial-resistant organisms are associated with higher rates of treatment failures, prolonged hospitalizations, increased costs and mortality (Ruiz *et al.,* 2018). Antimicrobial stewardship consists of avoidance of antimicrobials when appropriate and, when antimicrobials are indicated, use of strategies to optimize the selection, dosing, route of administration, duration and timing of antimicrobial therapy to maximize clinical cure while limiting the unintended consequences of antimicrobial use, including toxicity and selection of resistant microorganisms (Chen et al 2018). The *Citrobacter freundii* resisting beta-lactams by three main strategies the first one is hydrolyzing of beta-lactam ring by beta-lactamase enzymes, the encoding genes for beta-lactamases was plasmid-borne and easy to transfer from each other (Huang *et al.,* 2017). The second strategy is modifying the beta-lactams target (Penicillin-binding proteins), that leading to prevent

Table 6: Antimicrobials resistance profile of 30 *Citrobacter freundii* isolates from patients infected with urinary tract infection in Al-Najaf City-Iraq during the period from April to December 2018 (Contd….)

Isolate	Sex	Age group	No (%)	R	ESBL
1	F	51-60	12(80)	MDR	Positive
2	F	18-30	9(60)	MDR	Positive
3	F	41-50	13(86.6)	XDR	Positive
4	F	31-40	14(93.3)	XDR	Positive
5	F	51-60	14(93.3)	XDR	Positive
6	F	41-50	14(93.3)	XDR	Positive
7	M	51-60	14(93.3)	XDR	Positive
8	M	41-50	14(93.3)	XDR	Positive
9	F	41-50	12(80)	MDR	Positive
10	F	41-50	12(80)	MDR	Positive
11	M	31-40	12(80)	MDR	Positive
12	M	31-40	12(80)	MDR	Positive
13	M	31-40	14(93.3)	XDR	Positive
14	F	41-50	12(80)	MDR	Positive
15	F	18-30	12(80)	MDR	Positive
16	F	18-30	11(73.3)	MDR	Positive
17	F	41-50	12(80)	MDR	Positive
18	F	31-40	11(73.3)	MDR	Positive
19	F	41-50	13(86.6)	XDR	Positive
20	F	31-40	10(66.6)	MDR	Negative
21	F	31-40	6(40)	MDR	Negative
22	F	31-40	4(26.6)	MDR	Negative
23	F	51-60	3(20)	MDR	Negative
24	F	51-60	4(26.6)	MDR	Negative
25	F	41-50	4(26.6)	MDR	Negative
26	F	41-50	4(26.6)	MDR	Negative
27	M	41-50	4(26.6)	MDR	Negative
28	M	41-50	4(26.6)	MDR	Negative
29	M	51-60	4(26.6)	MDR	Negative
30	M	51-60	4(26.6)	MDR	Negative

AM: Ampicillin, AX: Amoxicillin, AMC: Amoxiclav, CTX: Cefotaxime, CRO: Ceftriaxone, CAZ: Ceftazidime, CN: Gentamicin, TM: Tobramycin, CIP: Ciprofloxacin, S: Streptomycin, E: Erythromycin, TE: Tetracycline, SSS: Sulfonamide, C: Chloramphenicol, F: Female, M: Male, No (%): Numbers and (percentages) of overall resistance, R: resistance type, MDR: Multi-drug resistance, XDR: Extensive-drug resistance, ESBL: Extended-spectrum beta lactamase producing bacteria.

antibiotics from binding to it. The third one is lowering the outer membrane permeability of antibiotics inside the cell and through it out of the cell by many mechanisms like efflux pumps (Juhas *et al.,* 2019). *Citrobacter freundii* phenotypically having the ability to resist aminoglycosides antibiotics due to many mechanisms like reducing uptake or decreasing cell permeability by a transport defect or membrane impermeable this mechanism is chromosomally mediated and results in cross-reactivity to all aminoglycosides (Brahmi *et al.,* 2018). The other mechanism is altered ribosome binding sites by mutations at the site of aminoglycoside attachment may interfere with ribosomal binding. Resistance to streptomycin can occur by this mechanism since this agent binds to a single site on the 30S subunit of the ribosome (Botts *et al.,* 2017). Resistance to the other aminoglycosides by this mechanism is uncommon since they bind to multiple sites on both ribosomal subunits and high-level resistance cannot be selected by a single step (Feng *et al.,* 2015). The most of isolates in this study showed an obvious resistance to chloramphenicol antibiotic. The target modifying may also contribute to chloramphenicol resistance, the efflux pump that is throwing away this antibiotic, causing decreasing of antibiotic concentration inside the bacterial cell (Anisimova and Yarullina 2018). The bacterial cell that has an ability to resisting the tetracycline antibiotic by many mechanisms such as hydrolyzing enzymes encoding by *tet*(X) genes, efflux pumps (A-E) types (Leski *et al.,* 2016). The bacterial cell can be resisting the sulfonamides due to the presence of an R-plasmid that determining sulfonamide-resistant di-hydropeteroate synthase (DHPS), DHPS enzymes

which hydrolyzing the sulfonamide antibiotics, the high resistance rate to this drug it's may be due the property of plasmid-bearing that lead to the spreading of resistant (Lee *et al.,* 2016). In this study showed the lower susceptibility rate to ciprofloxacin. The ciprofloxacin antibiotic determined as effective fluoroquinolone antibiotic against Enterobacteriaceae members but the resistance toward it is increasing, the resistance is due to many reasons like mutation in genes that encoding for gyrase enzymes alone or with mutation in genes that encoding to topoisomerase IV, another reason is excessive activity of efflux pumps, the mutations happing in regions called quinolone resistance-determining regions (responsible for quinolones resistance). In conclusion: *Citrobacter freundii* became is one of the most drugs resistance dangerous pathogens caused urinary tract infection in Iraq.

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