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Molecular Study of Some Virulence Factors and Antimicrobial Susceptibility Pattern of *Citrobacter frundii* Isolated from Human Diarrhea

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ABSTRACT

A total of (100) clinical specimens from Stool were collected under aseptic condition. These specimens were collected from patients attending to Al-Hilla Teaching Hospital in Babylon province from August (2019) and March (2020). Fecal samples were collected from (100) consecutive patients, with community-acquired gastro-enteritis. Quantitative biofilm formation experiments were performed in a microtiter (biofilm assay) using Trypticase Soy Broth supplemented with (1%) glucose. This assay was repeated as triplicate to increase the accuracy of assy. According to mean of value at (63) nm the results were interpreted as none, moderate and strong biofilm former when the mean of OD value were (<0.120, 0.120-0.240, and >0.240) respectively. The results revealed that seven isolates of *Citrobacter freundii* isolates were biofilm former (41.2%), the strong biofilm former were account for 5/7(71.4%) while isolates that express moderate biofilm formation were 1/7(14.3%), and 1/7(14.3%) isolates that express weak biofilm formation. The (17) *C. freundii* isolates were tested for susceptibility to (14) antibiotics belonging to (8) antibiotic classes using the disk diffusion method according to CLSI recommendations. Most of the (17) bacterial isolates were resistant to β-lactams, especially to Penicillins (35.29%), Cephalosporins (23.52%-70.58%). Resistance to the two quinolones (Ciprofloxacin and Levofloxacin) tested was (11.7%). Resistance to other antibiotics included Aminoglycosides (5.88%-11.7%), Tetracyclines (17.6%), Chloramphinicol (11.7%), Trimethoprim (23.52%) and Nitrofuran (23.52%). In this study, hemolysin production by all isolates were done, it was found that none of the isolates showed a typical hemolysis pattern on 5% sheep blood agar plates. Bacterial isolates were screened for siderophores production the results showed 16(94.1%) isolates were able to produce siderophores. Ten isolates were positive for protease production (58.8%). The prevalence of *viaB*, *hlyA*, *LT*, and *STp* related genes in the bacterial isolates were low in this study. DNA was extracted from all (17) isolates; conventional PCR was carried out using these DNA samples for the amplification of specific *viaB* primer; after that gel electrophoresis showed that, out of the (17) samples, only 7(41.2%) produced the specific (516) bp DNA fragment when compared with allelic ladder. A PCR survey was performed using primers *hlyA* to determine whether a (597) bp *hlyA* gene fragment could be detected in the (17) *Citrobacter freundii*. After that gel electrophoresis showed that, out of the (17) samples, only 1(5.9%) produced the specific (597bp) DNA fragment when compared with allelic ladder. After that gel electrophoresis to detect *TA* gene showed that, out of the (17) samples, 8(47.1%) Produced the specific (273bp) DNA fragment when compared with allelic ladder. Moreover, 4(23.5%) isolates produced the specific (166 bp) DNA fragment as recorded heat stable toxin gene among isolates in recent study.

Keywords: Virulence Factors of *Citrobacter frundii*, Antimicrobial Susceptibility, biofilm formation, virulence genes.

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INTRODUCTION

Citrobacter freundii is a Gram-negative, aerobic or facultative and rod shaped bacterium which is considered a commensal species of the intestinal tract of humans and other animals [Liu *et al*., 2016]. *Citrobacter freundii* is associated with several opportunistic infections, such as severe diarrhea, urinary tract infections, pneumonia, neonatal meningitis and brain abscesses in humans. Neonatal meningitis can be fatal with a 25-50% mortality rate among infants. However, *Citrobacter freundii* related infections are generally limited to infants and immuno-compromised patients [Pardia *et al*., 1989; Badger *et al*., 1999]. The virulence factors can be acquired by *Citrobacter freundii* strains that qualify them to cause different infections in humans. The major virulence factors found in diarrhea-associated *Citrobacter freundii* are toxins. According to Schmidt *et al*., [1993], *Citrobacter freundii* can acquire slt-II or slt-II related shiga-like toxins or capsular polysaccharide virulence antigen, such as *via B* gene, which is the most common virulence factor found in *Salmonella enteric serovar typhi* and *S. enteric serovar paratyphi* and causes typhoid-like diseases [Lin *et al*., 2007]. Other *Citrobacter* related virulence factors including proteolysis, hemolysis and biofilm formation have also been previously spotted [Fakruddin *et al*., 2014]. Biofilm formation was the important virulence factor among this bacteria in diarrheagenic [Pereira *et al*., 2010].

AIM OF THE STUDY

This study is aimed to identify and characterize the *Citrobacter freundii* in patients with diarrhea cases according to their virulence and antibiotic resistance patterns to determine the risk to public health, and detection of some virulence genes by PCR technique.

MATERIALS AND METHODS

A total of (100) clinical specimens from Stool were collected under aseptic condition. These specimens were collected from patients attending to Al-Hilla Teaching Hospital in Babylon province from August (2019) and March 2020. Fecal samples were collected from 100 consecutive patients, with community-acquired gastro-enteritis. Fecal samples (one per patient). After collection, each swab was immersed in 5 mL of saline (0.9%), mixed well by vortexing for 10 seconds, and centrifuged at 3,500 × g for 15 minutes. Most of the supernatant was decanted, and 100 µL of the sediment was inoculated directly on MacConkey agar and XLD agar (Oxoid Cambridge, UK) and incubated at 37°C for 24h. The morphological characteristics of the colonies including size, shape, color, were recorded, the suspected *Citrobacter* were relevant by biochemical test [McFadden, 2000], then finally confirmed by using Vitek-2 Compact (Bio Mérieux, France), (17) isolates were confirmed.

Biofilm Formation

Tissue culture plate method (TCP) assay (also called semi quantitative microtiter plate test (biofilm assay) described by [Christensen *et al*., 1985], was considered as standard test for detection of biofilm formation as follow:

1. Isolates from fresh agar plates were inoculated in tryptcase soya broth (TSB) containing 1% glucose an incubated anaerobically for 72 hrs, at 37°C and then diluted 1:100 with TSB.
2. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates' wells were filled with 150 µl of the diluted cultures and only broth served as control to check non-specific binding of media. Each isolate was inoculated in triplicate.
3. The tissue culture plates were incubated for 24 hrs, at 37°C. After incubation, content of each well was gently removed by tapping water. The wells were washed four times with phosphate buffer saline (pH7.2) to remove free-floating bacteria.
4. Biofilms formed by adherent sessile organisms in plate were fixed by placing in oven at 37°C for 30 minutes.
5. All wells stained with crystal violet (0.1 % v/v). Excess stain was rinsed off by through washing with deionized water and plates were kept for drying.
6. One hundred fifty (150) µl of acetone/ethanol (20:80, v/v) mixture was added to dissolve bounded crystal violet. The optical density (O.D.) at 570 nm was recorded and the results were interpreted according to the Table (1).

Table 1: Classification of bacterial adherence by TCP method

Mean of OD value at 630nm	Biofilm formation
$<OD_C$	Non-adherent
$OD_C < OD \leq 2 \times OD_C$	weakly adherent
$2 \times OD_C < OD \leq 4 \times OD_C$	Moderately adherent
$4 \times OD_C < OD$	Strongly adherent

Hemolysin Production

Hemolysin production was carried out by inoculating of blood agar medium with bacterial isolate at (37°C) for (24-48) hours, an appearance of clear zone around the colonies referred to complete hemolysis. Hemolysis (β-hemolysis) or greenish zone around the colonies referred to partial hemolysis (α- hemolysis), while no changing referred to non- hemolysis (γ- hemolysis) [Baron *et al*., 2003].

Siderophores Production

M_9 medium was prepared and then supplemented with (2%) agar agar after sterilization in autoclave and cooling to (50C°), (0.25) mg/L glucose (sterilized by filtration) and (200 mol/L) of dipyridyl were added. Then the organisms were inoculated into this media and it was incubated for (24 hours) at (37C°). The results were seen when the growth of organism was present or not [Nassif and philippe, 1996].

Extracellular protease production

This method was carried out by using M_9 media supplemented with (2%) agar agar. After sterilization in autoclave and cooling at (50C°), (0.25) mg/L glucose (sterilized by filtration) was added, and then, the media was supported by (1%) casein. After the inoculation of this media with bacterial strain and incubation for (24 - 48) hours at (37C°), (3ml) of Trichloroacetic acid (5%) was added to precipitate the protein. The positive result was read by observing a transparent area around the colony [Piret *et al*., 1983].

Genomic Bacterial DNA Extraction

Detection of virulence genes

The Polymerase Chain Reaction (PCR) was used to detect *via B*, *hlyA*, *LT*, and *STp* associated virulence genes in the isolated bacterial strains. The primers used were obtained from [Schmidt *et al*., 1983; Wong *et al*., 1997; Bo¨lin *et al*., 2006 and Sjo¨ling *et al*., 2007] are shown in Table (2). The PCR was conducted by Cosmo gene tech, Co., Ltd, Korea. The PCR mixture contained 0.5 µL of Taq polymerase, 10×SP Taq buffer, 2 µL of dNTP, 5.0 µL tuning buffer, 1.0 µL template and 12 µL dissolved water and 1 µL of each primer pairs. The amplification protocol consisted of 35 cycles of 0.5 min at 94ºC, annealing for 0.5 min at 62 ºC (*hlyA*) and 2 min extension at 72ºC with a 1 min final extension at 72ºC. PCR products were separated in 1.5% to 2% agarose gels and visualised under UV light. The thermal cycle for amplification of *via B* gene, *LT*, and *STp* consisted of 5 min initial denaturation at 95ºC, later 95ºC for 30 sec, 62ºC for anneal temperature up to 30 sec, 1 min elongation period at 72ºC and at last final extension was done at 72ºC for 10 min 5. The PCR products in the agarose gel were stained with ethidium bromide (0.5 mg mLG1) and observed. The final PCR bands were photographed after observation under ultraviolet light.

Table 2: DNA Genes Specific Primers which are used in PCR for Detection of Bacterial Isolates

Primers	Nucleotide sequence	Size of fragment (bp)	References
viaB	F-TGTCGAGCAGATGGATGAGCAT R-ACGGCTGAAGGTTACGGACCGA	516	[Schmidt *et al*., 1983]

hlyA	F-GGC CGG TGG CCC GAA GAT ACG GG R-GGC GGC GCC GGA CGA GAC GGG	597	[Wong *et al*., 1997]
LT	F-ACGGCGTTACTATCCTCTC R-TGGTCTCGGTCAGATATGTG	273	[Sjo¨ling *et al*., 2007]
STp	F-TCTTTCCCCTCTTTTAGTCAG R-ACAGGCAGGATTACAACAAAG	166	[Bo¨lin *et al*., 2006]

RESULTS AND DISCUSSION

Detection of biofilm formation

Quantitative biofilm formation experiments were performed in a microtiter (biofilm assay) using Trypticase Soy Broth supplemented with (1%) glucose. This assay was repeated as triplicate to increase the accuracy of assy. According to mean of OD value at (63) nm the results were interpreted as none, moderate and strong biofilm former when the mean of OD value were (<0.120, 0.120-0.240, and >0.240) respectively. The results revealed that seven isolates of *Citrobacter freundii* isolates were biofilm former (41.2%), the strong biofilm former were account for 5/7(71.4%) while isolates that express moderate biofilm formation were 1/7(14.3%), and 1/7(14.3%) isolates that express weak biofilm formation. These results were shown in Table (3). The results in this study indicates that the biofilm plays an important role in the establishment of *Citrobacter freundii* infection, enhanced pathogenesis and multi-drug resistance. In general, biofilm production is related to persistent infections which respond weakly to regular-antibiotic therapy. This helps to extent the antimicrobial resistant traits in nosocomial pathogens by expanding mutation rates and exchange of antimicrobial resistance genes [Niveditha *et al*., 2012]. Previous studies detected biofilm formation in clinical samples from patients [Zogaj *et al*., 2003]. One of the pathogenicity mechanisms of these bacteria is the formation of biofilms in their hosts, which contribute to an increase in the virulence of these microorganisms and in their resistance to antibiotics, consequently, in their survival.

Table 3: Production of biofilm in *Citrobacter freundii*

Bacterial isolate No.	Biofilm			
	Strong	Moderate	Weak	% of biofilm Formation
Citrobacter freundii 7(41.2%)	5(71.4%)	1(14.3%)	1(14.3%)	100%

Prevalence of Antimicrobial Resistance

The (17) *C. freundii* isolates were tested for susceptibility to (14) antibiotics belonging to (8) antibiotic classes using the disk diffusion method according to [CLSI, 2019] recommendations. Most of the (17) bacterial isolates were resistant to β-lactams, especially to Penicillins (35.29%), Cephalosporins (23.52% -70.58%). Resistance to the two quinolones (Ciprofloxacin and Levofloxacin) tested was (11.7%). Resistance to other antibiotics included Aminoglycosides (5.88%-11.7%), Tetracyclines (17.6%), Chloramphinicol (11.7%), Trimethoprim (23.52%) and Nitrofuran (23.52%). The results were shown in Table (4). Many isolates were multidrug resistant (MDR), with resistance to at least one antibiotic of three or more distinct classes (MDR ≥ 3). During the study, higher percentage of resistance was observed against β-lactam antibiotic, which may be due to production of β-lactamase. Since, all the isolates were susceptible towards carbapenems; it was assumed that this can be kept as reserve drug for the treatment of infections caused by *Citrobacter* isolates. The β-lactamase enzyme reacts with β-lactam bond and forms acyl enzyme intermediates, which undergoes rapid hydrolysis of β-lactam ring and results in the loss of antibiotic activity [Oliva *et al*., 2003]. According to Sharma *et al*., [2008], all bacteria produce at least one chromosomally mediated β-lactamase and these enzymes are specific for genus, species, and subspecies. It was observed a multi-resistant characteristic of *C. freundii* to antimicrobials Isolates were sensitive to Ciprofloxacin, Gentamicin, Tetracyclin and Chloranfenicol and Trimethoprim/Sulfamethoxazole. The bacterial multi-resistance is one of the most important problems concerning bacteria of medical importance like *C. freundii.* This bacteria has become increasingly resistant to a range of antibiotics [Liu *et al*., 2018]. Mohanty *et al*., [2007] reported that isolates of *C. freundii* isolates from patients in a tertiary care hospital of India had high degrees of resistance to Ceftazidime (85%), Cefotaxime (85%), Piperacillin (65%), and Ciprofloxacin (60%). In our study, (70.6.4%) resistant to second-generation Cephalosporins, such as Cefoxitin (23.5-58.82%) resistant to third-generation Cephalosporins and (23.5%) resistant to fourth-generation Cephalosporins such as Cefepime. *C. freundii* is often resistant to multiple classes of antibiotics, suggesting that both clinical and environmental strains may be a reservoir of antimicrobial resistance determinants [Yim *et al*., 2013; Feng *et al*., 2015; Leski *et al*., 2016 and Sheppard *et al*., 2016]. The MDR *C. freundii* strains have been associated with a higher rate of in-hospital mortality compared to susceptible strains [Leski *et al*., 2016]. In this study, we surveyed *C. freundii* from diarrheal patients, most isolates were resistant to more than three antibiotic classes out of the (8) distinct antibiotic classes tested. Seven MDR isolates were strongly produce biofilm. Such highly biofilm former MDR strains may cause more severe disease and their MDR properties may limit clinical therapeutic options when they cause disease.

Table 4: Antibiotics susceptibility test for *C. freundii* isolates

Antibiotics	No and % of isolates		
	Resistant (%)	Intermediary Resistant (%)	Sensitive (%)
PENICLLINS Ampicillin	6(35.29%)	7(41.17%)	4(23.52%)
CEPHALOSPORINS Cefotaxime Ceftazidime Cefepime Cefoxitin Ceftriaxone	 10(58.82%) 6(35.29%) 4(23.5%) 12(70.6%) 4(23.5%)	 5(29.41%) 7(41.17%) 12(70.6%) 4(23.5%) 7(41.2%)	 2(11.76%) 4(23.52%) 3(17.6%) 2(11.8%) 6(35.3%)
QUINOLONES Ciprofloxacin Levofloxacin	 2(11.7) 2(11.7)	 1(5.9) 2(11.7)	 14(82.4%) 13(76.6%)
AMINOGLYCOSIDES Gentamicin Amikacin	 1(5.9%) 2(11.7%)	 1(5.9%) 4(23.5%)	 15(88.2%) 11(64.7%)
TETRACYCLINES Tetracycline	 3(17.6%)	 0(0%)	 14(82.35%)
PHENICOLS Chloramphenicol	 2(11.7%)	 4(23.5%)	 14(82.4%)
SULFONAMIDES Trimethoprim/ Sulfamethoxazole	 4(23.5%)	 1(5.9%)	 12(70.6%)
NITROFURAN Nitrofurantoin	 4(23.5%)	 6(35.29%)	 7(41.17%)
Crpabenems Imepenem	 0(0%)	 1(5.9%)	 16(94.1%)

Detection of Hemolysin and Siderophores

In this study, hemolysin production by all isolates were done. It was found that none of the isolates showed a typical hemolysis pattern on 5% sheep blood agar plates. The lack of hemolytic inability indicates that our isolates could not break down red blood cells and cause hemolytic uremic syndrome in human [Narayanan *et al*., 2013]. On the other hand the bacteria which have no ability to produce this factor extracellulary may have further means to evade the action of WBCs or to prevent healing [Fakruddin *et al*., 2014]. Bacterial isolates were screened for siderophores production the results showed 16(94.1%) isolates were able to produce siderophores. The results were shown in Table (5). Iron is an essential element required by almost all bacteria. In the human organism, it is sequestered by iron withholding proteins, like transferrin, lactoferrin or ferritin, and is available only by means of a high-infinity iron uptake system. Hence, high- infinity iron uptake mechanisms are needed to proliferate successfully in the host [Schubert *et al*., 2002].

Detection of protease production

We found several virulence factors in the isolated *Citrobacter freundii*. Ten isolates were positive for protease production (58.8%), isolates showed proteolyticactivity on skim milk agar which allows them to hydrolyze the peptide bonds of essential proteins in eukaryotes, particularly in humans although the protease enzymes only infrequently serve as toxic factors to the host [Miyoshi, 2013].

Table 5: Some Virulence factors of *Citrobacter freundii* isoletes

Virulence factors	*Citrobacter freundii* n= 17	
	No. and (%) of positive isolates	No. and (%) of negative isolates
Hemolysin	0(0%)	17(100%)
Sidrophore	16(94.1%)	1(5.9%)
Extracellular protease	10(58.8%)	7(41.1%)

Molecular Study

The prevalence of *via B* and *hlyA*, *LT*, and *STp* related genes in the bacterial isolates were low in this study. DNA was extracted from all (17) isolates; conventional PCR was carried out using these DNA samples for the amplification of specific *viaB* primer; according to the sequences in Table

(2). After that gel electrophoresis showed that, out of the (17) samples, only 7(41.2%) produced the specific (516 bp) DNA fragment when compared with allelic ladder; as shown in Figure (1). The results of molecular detection for *viaB* gene for [Hossain *et al*., 2017] showed that (4) out of (17) samples gave positive results to *viaB* gene with a specific primer the incidence of this gene (23.5%). The presence of *viaB* gene allows the bacteria to evade the natural immune system by extending the host response during the infection [Rondini *et al*., 2017]. A PCR survey was performed using primers *hlyA* to determine whether a (597) bp *hlyA* gene fragment could be detected in the (17) *Citrobacter freundii*. After that gel electrophoresis showed that, out of the (17) samples, only 1(5.9%) produced the specific (597bp) DNA fragment when compared with allelic ladder. Rondini *et al*., [2017] showed no positive bands on PCR for *hlyA* gene among isolates of *Citrobacter freundii.* Previous reports show that some strains of *C. freundii* scarry Shiga toxins, heat-stable toxins, and cholera toxin homologs (*cfxAB*) [Guarino *et al*., 1987; Tschape, 1995 and Karasawa *et al*., 2002]. Our study detect for the presence of Shiga toxin genes by PCR using primers reported previously, for heat stable and heat labile enterotoxins using primers, and for cfxAB using primers designed based on sequences from *C. freundii* 09-1 [Zhang *et al*., 2002 and Rodas *et al*., 2009]. After that gel electrophoresis to detect *TA* gene showed that, out of the (17) samples, 8(47.1%) Produced the specific (273bp) DNA fragment when compared with allelic ladder as shown in Figure (2). Moreover, 4(23.5%) isolates produced the specific (166 bp) DNA fragment as recorded heat stable toxin gene among isolates in recent study as shown in Figure (3). It was reported that the Shiga toxin (heat labile and heat stable) genes had been detected in *C. freundii* [Schmidt *et al*., 1983 and Herold *et al*., 2004]. These observations allow us to draw a possible parallel with the emergence of Shiga toxin-producing biofilm and aggregative *C. freundii*. The current study was similar with study that conducted by [Li *et al*., 2012] who found Shiga toxin like among *Citrobacter* comprised low percentage. Results are not surprising considering that the prevalence of these toxin genes in *C. freundii* is likely to be low. There is also clear evidence of the presence of shiga like toxins in some strains of *C. freundii*. Overall, the isolated strains of *C. freundii* that expressed virulent factors detected through conventional and PCR methods have the potential to act as human pathogens.

CONCLUSION

This study concluded that *citrobacter freundii* could be one causes of gastrointestinal tract infections. The virulence factors and antimicrobial resistance patterns of this bacteria expose significant public health risk factors. Molecular study showed low frequencies of shiga like toxins among isolates included in the recent study.

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Figure 1: Agarose gel electrophoresis (1.5%) of RCR amplified of *viaB* gene (516) bp of *Citrobacter frundii* for (55) min at (70) volt

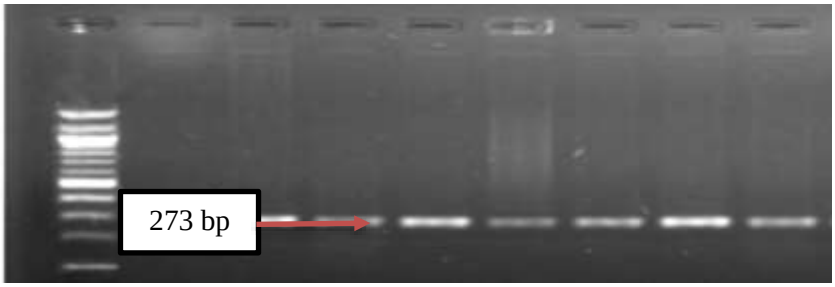

Figure 2: Agarose gel electrophoresis (1.5%) of RCR amplified of *LT* gene (273) bp of *Citrobacter frundii* for (55) min at (70) volt

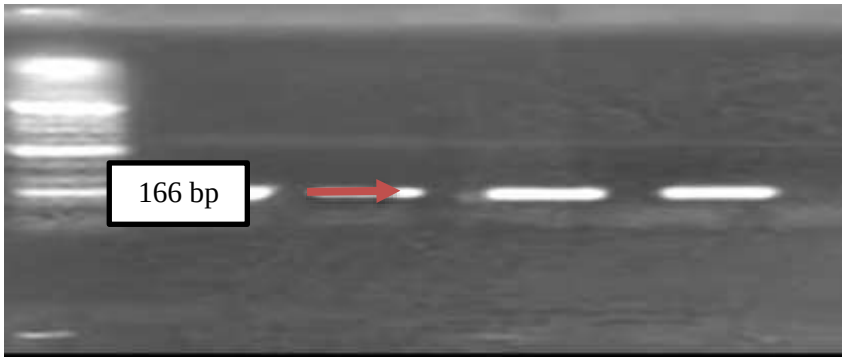

Figure 3: Agarose gel electrophoresis (1.5%) of RCR amplified of *STp* gene (166) bp of *Citrobacter frundii* for (55) min at (70) volt