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Antimicrobial susceptibility patterns and virulence genes detection in *Citrobacter freundii* isolated from patients at a tertiary care hospital in Bangladesh

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Abstract

Background: *Citrobacter freundii* is an infrequent hospital-acquired pathogen causing different types of disease in clinical settings. This pathogen is associated with a wide range of infections causing an unpredictably high mortality rate of 30-60%. Separation of this pathogen in health care settings is escalating and multidrug-resistant strains are emerging. Therefore, this study aimed to detect antimicrobial resistance patterns and virulence genes among the isolated *C. freundii.*

Methods: A total of 500 samples (urine, stool, wound swab and pus, blood endotracheal aspirates, and sputum) from patients with clinically suspected infections irrespective of age and sex were used in this study. Disc diffusion method was used to detect the susceptibility pattern of antibiotics; colistin, tigecycline, and fosfomycin susceptibility pattern was identified by the minimum inhibitory concentration (MIC) method. Polymerase chain reaction (PCR) was done to detect potential virulence genes.

Results: Among 27 isolated *C. freundii*, the majority were resistant to amoxiclav (92.59%), trimethoprim-sulfamethoxazole (88.89%), cefotaxime, and ceftazidime (85.19%) followed by ceftriaxone (81.48%), cefepime and ciprofloxacin (77.78%). MIC showed the least resistance to colistin (29.63%), fosfomycin (11.11%) and tigecycline (7.41%). Though major virulent genes present in *C. freundii* are *lt, lt-A, lt-h,slt-I,slt-II, via B, hly A*, but not all genes are always commonly detected in *C. freundii*. We studied the common genes which were also detected in previous years. In our study, among the common genes, PCR was

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positive for *via B* gene (48.15%) and lt-A gene (25.93%). *hly A, lt, lt-h* genes showed negative results

Conclusion: Antibiotic resistance found in this study is quite worrisome as widespread resistance is increasingly seen among the bacteria isolated from human infection. Additionally, virulence genes play important role in *C. freundii* infection.

Keywords
Antimicrobial Resistance, *Citrobacter Freundii*, Nosocomial Infection, Virulence Gene.

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Introduction

Citrobacter freundii is rod-shaped, aerobic or facultatively anaerobic, gram-negative bacilli which is a commensal inhabitant of intestinal tracts of humans and other animals [1]. Being an opportunistic pathogen, it is responsible for a number of infections like severe diarrhea, urinary tract infection, wound infection, bone and soft tissue infection, bloodstream infection and septicemia [2]. A prolonged period of hospital stay causes more vulnerability to *C. freundii* infection since this organism is one of the important causes of nosocomial infection [3]. Virulence is inherently coupled to disease-causing morbidity and mortality [4]. Eventually, they obtain integrons, transposons or plasmids encompassing virulence factors and resistance genes that alter these bacteria into further virulent and resilient organisms [5]. Some acquired virulence traits are considered to be responsible for food poisoning and diarrhea [6]. Among them, toxins namely Shiga-like toxin, heat-stable toxin, heat-labile toxin, and cholera toxin B subunit homolog [7] are the major virulence factors of *C. freundii* [8]. It can also attain virulence antigens such as capsular polysaccharides, via the B gene, which is said to be one of the utmost shared virulence factors found in typhoid-like diseases caused by *Salmonella* enteric serovar Typhi and Paratyphi [9]. Another virulence factor of C. freundii is hemolysin, encoded by *hlyA* gene, which is responsible for damaging tissues as well as bacterial dissemination [10].

In recent decades, a rising problem for public health is the appearance and extent of antimicrobial resistance among pathogenic bacteria [11]. Increased selective pressure in the hospital environment may help *C. freundii* to procure compound resistance factors for becoming multidrug-resistant and set up a momentous source of innumerable hospital-acquired infections [12].

Few studies have investigated the occurrence of the antibiotic resistance pattern of *C. freundii* in Bangladesh. But till now no virulence factor of *C. freundii* has been identified in Bangladesh. Hence, this study was aimed at detecting antimicrobial resistance patterns along with virulence genes.

Materials and Methods

Sample collection

This cross-sectional study was conducted in the Department of Microbiology at Dhaka Medical College, Dhaka from July 2019 to June 2020 for a period

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of one year. A total of 500 samples (urine, stool, wound swab, blood, pus, endotracheal aspirate, and sputum) were included in this study. Following the aseptic technique, all samples were collected and transported instantly to the Microbiology

Laboratory for further processing

Inclusion criteria

Patients with clinically suspected infections who got admitted to Dhaka Medical College Hospital (DMCH) were included for the collection of samples of urine, stool, wound swab, pus, sputum, and blood. Endotracheal aspirate samples were collected from patients who had suspected clinical infections and mechanical ventilation for more than 48 hours in the Intensive Care Unit (ICU) of DMCH. All these samples were collected irrespective of sex and antibiotic intake. Samples received in the microbiology department for culture and sensitivity were also included after taking informed written consent.

Isolation and identification of bacteria

All the urine, wound swab, pus, endotracheal aspirate, and sputum samples were streaked onto blood agar and MacConkey agar media and incubated for 24 hours aerobically at 370C. For blood samples, trypticase soy broth was used for primary culture at 370C for 24 hours, and then, for subculture, both blood agar and MacConkey agar media were used. Stool samples were streaked onto MacConkey agar media. After overnight incubation, the plates were examined for presence of any bacterial colony. Biochemical tests (Triple Sugar Iron, Citrate, Motility Indole Urease, Methylene Red- Voges Proskauer) were done to detect C. freundii. The C. freundii strains were inoculated in microcentrifuge tubes containing trypticase soya broth, kept for overnight incubation. Following that, the tubes were centrifuged at 4000 x g, supernatant was discarded. Then the pellets were stored at -200 C for extraction of DNA. Virulence genes were identified by PCR using primers.

Antibiotic susceptibility testing

The Kirby-Bauer modified disc-diffusion technique was used to perform antibiotic susceptibility patterns as described by the Clinical and Laboratory Standards Institute (CLSI, 2020). Antibiotic discs of amoxiclav (amoxicillin 20µg and clavulanic acid 10µg), piperacillin-tazobactam (100/10µg), Trimethprim-sulfamethoxazole (trimethoprim 1.25µg and sulfamethoxazole 23.75µg), ceftriaxone (30µg), cefoxitin (30µg), cefotaxime (30µg)ceftazidime (30µg), cefepime (30µg), ciprofloxacin (5µg), amikacin (30µg) and imipenem (10µg) were used (Oxoid Ltd. UK). For antibiotic susceptibility testing, Meuller-Hinton agar media was used. Resistant and sensitive bacteria were defined according to CLSI guidelines, 2020. MIC of colistin, tigecycline, and fosfomycin was carried out using the agar dilution method based on EUCAST and FDA guidelines, 2020. As a quality control strain, *E. coli* ATCC25922 was used.

Genomic PCR

Extraction of DNA

Three hundred microliters of distilled water were mixed with pelleted bacteria and vortexed. Then, in a heat block (DAIHA Scientific, Seoul, Korea), tubes were kept boiling at 100°C for 10 minutes. Next, tubes were kept on ice immediately. Following that, the tubes were centrifuged of the tubes for 6 minutes at 14000 x g at 4°C. Lastly, supernatants were transferred to another microcentrifuge tube via pipette, and these products were used as DNA templates for PCR. A temperature of -20°C was used to preserve these DNA templates for upcoming use.

Primer and master mix mixing with DNA template

A volume of 25 µl was used PCR reaction mixture. Every tube of PCR had 1 µl forward primer, 1µl reverse primer, 2 µl of extracted DNA, 9 µl master mix (mixture of reaction buffer, taq polymerase enzyme, $MgCl_2$, dNTP), 2 µl of extracted DNA, and

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12 µl nuclease-free water. After a short-term vortex, centrifugation was done for a few seconds.

Amplification through thermal cycler. PCR amplification assay was done in a Thermal cycler (Eppendorf AG, Master cycler gradient, Hamburg, Germany). All PCR runs are as follows: denaturation for 10 minutes at 94°C followed by 36 cycles at 94°C for one minute then annealing at 58°C for 45 seconds and final extension at 72°C for 2 minutes with finishing extension at 72°C for 10 minutes.

Gel electrophoresis and observation of bands

Using 1.5% agarose (Bethesda Research Laboratories) PCR product bands were detected by running amplified products onto horizontal gel electrophoresis with 1X TBE buffer for 30 minutes at 100 volts (50 mA). Loading dye was mixed with five µl of amplified DNA and placed on the gel into a separate well. Comparison of base-pair of the identified band was done with one hundred bp molecular size DNA ladder which was put into another well of the gel. Then, at room temperature, ethidium bromide (0.5 µl/ml) staining for 30 minutes and distaining for 15 minutes with distilled water were done to spot the DNA bands. UV trans-illuminator (Gel Doc, Major science, Taiwan) was used to observe the bands, and the photograph was taken using a digital camera.

Statistical analysis

Systemic documentation of all results of the study was done. Analysis of data was completed by using the 'Microsoft Office Excel 2010' program and SPPSS version 10.

Ethical clearance

This study was approved by Research Review Committee (RRC) and Ethical Review Committee (ERC) of Dhaka Medical College with ethical board approval number: ERC-DMC/ECC/2020/10 (R) on 08/01/2020.

Results

Citrobacter freundii may play an exclusive part in bacterial progression. Due to low virulence, they may remain in a host population for elongated periods. Low virulent antibiotic-resistant *Citrobacter* spp. is common colonizers of immunosuppressed patients who are exposed to multiple antibiotics. Also, the presence of low virulence resistant bacteria can complicate surveillance and infection control efforts *[20]. In this study, virulence genes viaB and lt*-A were detected in 48.15% and 25.93% of isolated *C. freundii* respectively. This finding is in agreement with the findings in Iraq which reported the presence of these genes (*viaB* and *lt-A*) in 41.2% and 36.36% of isolated *C. freundii* respectively [21,22]. There is no data available regarding the prevalence of virulence genes in *C. freundii* in Bangladesh or nearby Asian countries. **(Table 1)**

Table 1. Detection of antimicrobial susceptibility pattern of *C. freundii* (N=27).

Antimicrobial Agent	Sensitive		Resistant	
	n	%	n	%
Amoxiclav	2	7.41	25	92.59
Pipercillin-tazobactam	8	29.63	19	70.37
Trimethoprim-sulfomethoxazole	3	11.11	24	88.89
Cefoxitin	5	18.52	22	81.48
Cefotaxime	4	14.81	23	85.19
Ceftazidime	4	14.81	23	85.19
Ceftriaxone	5	18.52	22	81.48
Cefepime	6	22.22	21	77.78
Aztreonam	4	14.81	23	85. 19
Ciprofloxacin	6	22.22	21	77.78
Imipenem	18	66.67	9	33.33
Doxycycline	8	29.63	19	70.37
Amikacin	9	33.33	18	66.67
Colistin	19	70.37	8	29.63
Fosfomycin	24	88.89	3	11.11
Tigecycline	25	92.59	2	7.41

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Table 2. Primers used for detection of virulence genes of *C. freundii* (N=27).

Genes	Primer	Sequence	Amplicon	Ref.
via B	F	TGTCGAGCAGATGGATGAGCAT	516	13
	R	ACGGCTGAAGGTTACGGACCGA		
hly A	F	GGCCGGTGGCCCGAAGATACGGG	597	14
	R	GGCGGCGCCGGACGAGACGGG		
lt	F	AGCAGGTTTCCCACCGGATCACCA	132	15
	R	GTGCTCAGATTCTGGGTCTC		
lt-A	F	GGCGACAGATTATACCGTGC	696	15
	R	CCGAATTCTGTTATATATGTC		
lt-h	F	GCGTTACTATCCTCTCTATG	320	15
	R	ATTGGGGGTTTTATTATTCC		

Table 3. Distribution of virulence genes among isolated *C. freundii* in different samples (N=27).

Genes	Urine/	Wound and pus/	Sputum/	Endotracheal aspirate	Blood/	Stooln	Total
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
*viaB**	6(2+4**) (22.22)	2 (7.41)	0	1 (3.70)	2 (7.41)	2(1+1**) (7.01)	13 (48.1)
hlyA	0	0	0	0	0	0	0
lt	0	0	0	0	0	0	0
lt-A**	6 (2+4*) (22.22)	0	0	0	0	1* (3.70)	7 (25.93)
lt-h	0	0	0	0	0	0	0

*: Denotes positive for via B gene, **: Denotes positive for lt-A gene.

Virulence genes were detected using specific primers **(Table 2)**

Among the *detected viaB and lt-A* genes, 4 out of 6 urine samples contained both *viaB and lt-*A genes and one of 2 of stool samples was positive for both *viaB* and *lt-A* genes. **(Table 3)**.

Discussion

Nosocomial infections are considered the most important apprehension of public health these days which results in extensive mortality and morbidity for hospitalized patients [13]. *C. freundii* is alleged to be the most common nosocomial pathogen with a prevalence rate of 50% [14]. The excessive mortality rate by Citrobacter infection may possibly be due to undifferentiating use of antibiotic therapy since they are often resilient to regularly used antibiotics. Increasing use of cephalosporins, in particular the 3rd generation cephalosporins, have also added to the progression of extended-spectrum beta-lactamase (ESBLs) production and MDR [15].

In this study, *C. freundii* seemed hig*hly* resistant to amoxiclav (92.59%), trimethoprim-sulfamethoxazole (88.89%), 2nd, 3rd and 4th generation cephalosporin (85.19%) and ciprofloxacin (77.78%). The suppositions of the present study match with the former study results [16, 7, 17]. Some *Citrobacter*

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spp. isolates contain chromosomally mediated ß-lactamase like cephalosporinases and penicillinase which result in the occurrence of drug resistance and treatment failure regardless of initial susceptibility [18]. Resistance to cephalosporin may be due to decreasing membrane permeability on membrane protein purines [19].

C. freundii may play an exclusive part in bacterial progression. Due to low virulence, they may remain in a host population for elongated periods. Low virulent antibiotic-resistant *Citrobacter* spp. is common colonizers of immunosuppressed patients who are exposed to multiple antibiotics. Also, the presence of low virulence resistant bacteria can complicate surveillance and infection control efforts [20]. In this study, virulence genes *viaB* and *lt-A* were detected in 48.15% and 25.93% of isolated *C. freundii* respectively. This finding is in agreement with the findings in Iraq which reported the presence of these genes (*viaB* and *lt-A*) in 41.2% and 36.36% of isolated *C. freundii* respectively [21, 22]. There is no data available regarding the prevalence of virulence genes in *C. freundii* in Bangladesh or nearby Asian countries.

Conclusion

Efforts should be given to increase the detection and control of *C. freundii* infection. This study represents a low level of virulence in *C. freundii* along with higher resistance rates toward most tested antibiotics but lower for imipenam, colistin, fosfomycin, and tigecycline.

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Nil.

Competing interest

The authors declare that they have no competing interests.

Abbreviations

MIC, Minimum inhibitory concentration; PCR, Polymerase chain reaction; ICU, Intensive care unit; TSI, Triple sugar iron; MUI, Motility indole urea; MR-VP,Methyl Red-Voges-Proskauer; CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; FDA, Food and Drug Administration; ATCC, American Type Culture Collection; MgCl2, Magnesium chloride; dNTP, Deoxynucleotide triphosphate; TBE, Tris-borate-EDTA; DNA, Deoxyribonucleic acid; UV, Ultraviolet.

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