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The functional repertoire of *AmpR* in the *AmpC* β-lactamase high expression and decreasing β-lactam and aminoglycosides resistance in ESBL *Citrobacter freundii*

Falak Naz Tariq [a], Mehreen Shafiq [a], Nadeem Khawar [b], Gul Habib [a,*], Haji Gul [c], Azam Hayat [a], Mujaddad Ur Rehman [a], Ihab Mohamed Moussa [d], Eman A. Mahmoud [e], Hosam O. Elansary [f]

[a] *Department of Microbiology, Abbottabad University of Science and Technology, Havelian, Abbottabad, 22500, Pakistan*
[b] *Institute of Pathology and Diagnostic Medicine, Khyber Medical University, Peshawar, 25000, Pakistan*
[c] *College of Animal Science and Technology, Anhui Agricultural University, No. 130 Changjiang West Road, Hefei, 230036, China*
[d] *Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh, 11451, Saudi Arabia*
[e] *Department of Food Industries, Faculty of Agriculture, Damietta University, Damietta, Egypt*
[f] *Department of Plant Production, College of Food & Agriculture Sciences, King Saud University, P.O. Box 2460, Riyadh, 11451, Saudi Arabia*

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ABSTRACT

Citrobacter freundii is characterized by *AmpC* β-lactamases that develop resistance to β-lactam antibiotics. The production of extended-spectrum β-lactamase (ESBL) is substantially high in *Escherichia coli*, *C. freundii, Enterobacter cloacae*, and *Serratia marcescens*, but infrequently explored in *C. freundii*. The present investigation characterized the ESBL *C. freundii* and delineated the genes involved in decrease in antibiotics resistance. We used the VITEK-2 system and Analytical Profile Index (API) kit to characterize and identify the *Citrobacter* isolates. The mRNA level of *AmpC* and *AmpR* was determined by RT-qPCR, and gel-shift assay was performed to evaluate protein-DNA binding. Here, a total of 26 *Citrobacter* strains were isolated from COVID-19 patients that showed varying degrees of antibiotic resistance. We examined and characterized the multidrug resistant *C. freundii* that showed ESBL production. The RT-qPCR analysis revealed that the *AmpC* mRNA expression is significantly high followed by a high level of *AmpR*. We sequenced the *AmpC* and *AmpR* genes that revealed the *AmpR* has four novel mutations in comparison to the reference genome namely; Thr64Ile, Arg86Ser, Asp135Val, and Ile183Leu while *AmpC* remained intact. The ΔAmpR mutant analysis revealed that the *AmpR* positively regulates oxidative stress response and decreases β-lactam and aminoglycosides resistance. The *AmpC* and *AmpR* high expression was associated with resistance to tazobactam, ampicillin, gentamicin, nitrofurantoin, and cephalosporins whereas *AmpR* deletion reduced β-lactam and aminoglycosides resistance. We conclude that *AmpR* is a positive regulator of *AmpC* that stimulates β-lactamases which inactivate multiple antibiotics.

* Corresponding author.
E-mail addresses: gulhabib101@gmail.com (G. Habib), imoussa1@ksu.edu.sa (I.M. Moussa), helansary@ksu.edu.sa (H.O. Elansary).

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1. Introduction

The emergence of multidrug resistant bacteria in healthcare sectors presents a serious challenge to public health and clinicians [1]. The β-lactam antibiotics are widely used in hospitalized patients and resistance develops among microbes because of the β-lactamase enzyme production [2]. Various clinical isolates have become a global health concern because of β-lactam resistant strains including *C. freundii*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumonia*, and *Serratia marcescens* which are equipped with β-lactamases [3,4]. The genus *Citrobacter* belongs to the family of *Enterobacteriaceae* and members are found in soil, water, and sewage. *Citrobacter* can colonize human and animal gastrointestinal tracts and is widely associated with nosocomial infections [5]. *C. freundii* develops resistance to antibiotics by several mechanisms including the production of β-lactamases that hydrolyze the β-lactam ring of antibiotics. The β-lactamases have been classified by Ambler and Bush-Jacobi-Medeiros based on their molecular structure and functional similarities. Ambler classified β-lactamases into four classes; A, C, and D classes which use a serine whereas class B uses the metal zinc as an enzyme active center [6]. In the updated Bush-Jacobi-Medeiros classification, β-lactamases are classified into three groups based on the functional similarities between substrate and inhibitor profiles [7]. Group 1 commonly known as cephalosporinases consist of Ambler class C β-lactamases (AmpC enzymes). This group involves chromosomal and plasmid encoded genes that code for ESBL enzymes. The enzymes are induced by β-lactam antibiotics and the bacteria showed resistance to penicillin, cephamycin, tazobactam, and cephalosporins but were sensitive to cefepime and carbapenems. The well-known example of group 1 β-lactamases genes include *AmpC* of *E. coli* , *CMY-2* of *E. cloacae* , and *blaCMY* of *C. freundii* that reduce the effectiveness of β-lactam antibiotics by conferring resistance to the narrow and broad-spectrum antibiotics. Group 2 commonly known as serine β-lactamases, has serine in the active center and includes Amber classes A and D. Group 3 comprises metallo-β-lactamases that include class B of Amber classification [7,8]. Within the genome of *C. freundii*, the β-lactamase *blaCMY* (*AmpC*) genes are present that produce β-lactamases [9]. The *AmpC* β-lactamase is regulated by the *AmpR* which is located upstream of the *AmpC* [10,11]. The *AmpR* mutant showed low expression of *AmpC* whereas constitutive expression of *AmpR* resulted in high production of *AmpC* [12]. The inducible vector encoded *AmpC* β-lactamases with *AmpR* have been studied in *E. cloacae*, *Morganella morganii*, and *C. freundii*. For instance, ACT-1, DHA-1, and CMY-13 are plasmid-encoded β-lactamases and their inducible expression gave resistance to cephalosporins in *E. cloacae* [13], *M. morganii* [14], and *C. freundii* [15] respectively. Previous reports have shown that most of the Gram negative bacilli such as *E. coli* reconstitute and recycled their cell wall from cell wall degraded products such as anhydromuropeptides [16]. In *C. freundii*, the presence of β-lactam antibiotic increased the cytoplasmic anhydromuropeptides which bind to *AmpR* and activated *AmpC* while the absence of activating ligand like peptidoglycan recycled product repressed *AmpC* expression [17,18]. For example in *E. coli*, unhydrolyzed anhydromuropeptide accumulation in the cytoplasm activated *AmpR* which induced *AmpC* expression [19]. Previously, Ryuichi and coworkers determined the *AmpC* β-lactamase expression level with or without *AmpR* and their results suggested that the resistance of CFE-1 (plasmid-encoded *AmpC* β-lactamase) to cephalosporins is due to the substitution of Asp135Ala in *AmpR* of *C. freundii* [20]. Among the 13 *Citrobacter* species, *C. freundii* is considered an opportunistic pathogen that colonizes the respiratory and urinary tract of hospitalized patients [21]. To date, there is no comprehensive report of *Citrobacter* infection in COVID-19 patients, and the present report delineated an ESBL *C. freundii* that showed multidrug resistance profile from hospitalized patients. We hypothesized the involvement of *AmpC* and *AmpR* genes in the development of β-lactam resistance and speculated new mechanism of resistance. Initially, the strains were identified by the VITEK-2 system and characterized by an API test kit. We analyzed the relative expression levels of *AmpC* and *AmpR* and examined the effects of *AmpR* on the expression of *AmpC*. We aim to elucidate the mechanism by which *C. freundii* acquired β-lactam and aminoglycosides resistance and corroborated the role of *AmpR* in the production of ESBL.

2. Materials and methods

2.1. Samples processing and identification

We collected bacterial samples from COVID-19 patients between the years 2020 and 2021 and screened 3492 samples and detected bacterial coinfection and superinfection in hospitalized patients [22–24]. The sources of specimens were nasopharyngeal and endotracheal swabs. The bacterial isolates from COVID-19 patients that were resistant to antibiotics were refreshed from −20 °C and cultured on tryptic soy agar (TSA) and Luria Bertani (LB) agar. The VITEK-2 system and 16 S rRNA sequencing were used for the characterization and identification of the isolates. Briefly, the isolates were subcultured on TSA plates, and the bacterial culture turbidity was adjusted with a densitometer to the McFarland 0.5 standard in normal saline. The VITEK-2 identification Gram-negative bacterial cards were loaded into the VITEK-2 system and the test cards were automatically filled with a bacterial suspension and incubated for 3 h. The cards were read by kinetic fluorescence measurement every 15 min and the data was analyzed by the VITEK-2 system software and the results were generated automatically [25]. Afterward, the reference identification and characterization were done with the API 20E system (bioMérieux, Inc., France). Next, the MICs of *C. freundii* to tazobactam, ampicillin, gentamicin, ciprofloxacin, cefotaxime, and azithromycin were determined by broth microdilution method according to Clinical and Laboratory Standards Institute guidelines as previously described [26,27].

2.2. Growth conditions and culture media

C. freundii can grow on tryptic soy broth, LB broth, and Muller-Hinton (MH) agar (Sigma-Aldrich, Darmstadt, Germany). The antibiotics were purchased from the local market and stock solutions of antibiotics were preserved at −20 °C. The MIC was performed

on cation-adjusted MH broth and the antibiotic disc assay was performed on TSA as previously described [27,28]. For the API 20E test kit, *C. freundii* 24 h culture suspension was made in normal saline and inoculated to each test tube in the kit strip. The strips were incubated at 37 °C for 24 h and seven digits code was generated according to the manufacturer's instructions.

2.3. API 20E test kit protocol

The API 20E test strip consists of twenty tests reaction that are widely used for the identification of *Enterobacteriaceae*. The *C. freundii* colonies were suspended in normal saline and inoculated into an API 20E test strip. The test tubes ADH, URE, ODC, H_2S, and LDC tubes were overlaid with mineral oil to concede the anaerobic reactions whereas the CIT, GEL, and VP were filled with saline suspension. The strip was incubated at 37 °C for 24 h and the test reagent such as ferric chloride was added to TDA, Kovacs reagent to IND, and one drop of VP reagent 1(40% KOH) and VP Reagent 2 (α-naphthol) to VP were added. The color change was observed and the seven digits code was generated from the reactions and evaluated by the API catalog.

2.4. ESBL production assay

ESBL production was tested by double disc assay comprising cephalosporin (cefotaxime 30 μg) alone and cephalosporin plus clavulanic acid (30 μg). The zone of inhibition was compared between the single antibiotic disk (cefotaxime) and combination disks (cefotaxime plus clavulanic acid) and the zone $\geq$ 5 mm for the combination disks relative to the single antibiotic disk was treated as ESBL positive [29]. The gentamicin (CN10μg), tobramycin (TOB10μg), tazobactam (TZP110μg), ampicillin (AMP30μg), nitrofurantoin (F110μg), ciprofloxacin (CIP2μg), cefotaxime (CTX30μg), and cefotaxime plus clavulanic acid (CTX-CVA30μg) discs were used to check the resistance pattern of *C. freundii*.

2.5. Genome extraction, PCR, and gene sequencing

C. freundii strain was cultured in LB broth at 37 °C and the genomic DNA was extracted from overnight culture by Thermo Scientific DNA Purification Kit. The genomic regions of *AmpR* and *AmpC* were amplified from the genomic DNA of *C. freundii* using the primers listed in Table 1. Primers were constructed from the available genome sequence of *C. freundii* (NCBI accession number NZ_CP033744.1). The PCR program consists of the following steps; step1-initial denaturation at 95 °C for 5 min; step2-denaturation at 95 °C for 30 s, primer annealing at 55 °C for 1 min or 50 s, extension at 72 °C for 1 min, 35 cycles; step3-final extension at 72 °C for 5 min.

2.6. RNA extraction and RT-qPCR

C. freundii overnight culture was harvested, washed with ddH_2O, and dissolved in 1 ml of RNAiso Plus. The cell lysis was performed by 0.1-mm silica beads and the lysates were centrifuged at 12,000 rpm for 30 min. Further, the extract was treated with DNase I enzyme for residual DNA removal and the purity of RNA was determined from the ratio of absorbance at 260/280 nm. Reverse transcription and qPCR were performed by PrimeScript 1st strand cDNA synthesis kit and SYBR Premix Ex-Taq reagent kit respectively. The RT-qPCR analysis was performed by StepOne real-time PCR system (Applied Biosystems, USA). The gene signal was normalized

Table 1
All the primers and plasmids used in this study are listed in table.

Strain	Relevant genotype	Source
C. freundii ESBL	Clinical isolate	Hospital
ΔAmpR	*C. freundii AmpR* mutant	This study
E. coli DH5α	Transformation	TransGen
E. coli BL21	Protein expression	TransGen
Plasmids		
pKD4	Template for FLP recognition target site, Kan^r, Amp^r	[26]
pKD46	Temperature-sensitive, λ Red recombinase plasmid, Kan^r, Amp^r	[26]
pCP20	Temperature-sensitive, FLP recombinase plasmid, Amp^r, Cm^r	[26]
pET28a (+)	Recombinant protein expression in *E. coli* BL21, Kan^r	Novagen
Primer name	**Oligonucleotide (5′-3′)**	**Application**
AmpR- forward-1	AGGCTTAATGATGACGCGTAGCTATATCCCTCTGTAGGCTGGAGCTGCTTC	AmpR mutant
AmpR-reversed-2	GTTAATTATCAGGCCCGCCATTGGCGGGCCTTTTCATATGAATATCCTCCTTAG	AmpR mutant
AmpR-forward-3	ATGACGCGTAGCTATATCCCTCTTAAC	PCR
AmpR-reversed-4	GTGCAGCACCCCGGTCAACCAACGGGA	PCR
AmpR-forward-rt1	GTGACGCATTCTGCCATCAGCCAGC	RT-qPCR
AmpR-reversed-rt2	TTCCTGGGTCTGTTTAGTGGCAA	RT-qPCR
AmpC-forward-7	TGATTTCATGATGAAAAAATCGATATGC	PCR
AmpC-reversed-8	CAGTTATTGCAGTTTTTCAAGAATGCGC	PCR
AmpC-forward-rt3	GAGCAGGCTATTCCGGGCATGGCCG	RT-qPCR
AmpC-reversed-rt4	ATAGCGTCGCCGCCCAACACGCCGT	RT-qPCR

with *rpoD* gene cDNA abundance. All primers are listed in Table 1.

2.7. Electrophoretic mobility shift assay (EMSA)

EMSA was performed to detect AmpR and AmpC proteins binding to the promoter region. Initially, AmpR and AmpC proteins were expressed in pET28a (+) vector in *E. coli* BL21, and purified through Ni^{+2}-NTA histidine affinity column. The biotin-labeled probe (60bp) was incubated with AmpR and AmpC protein separately for 1 h at 37 °C. The 12 μl reaction mixture contains probe (3 fmol), protein (8 pmol), and lysis buffer. The reaction was stopped by heating at 95 °C for 5 min. The sample was run on 4% native polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer at 400 mA for 30 min. A nylon membrane was used to transfer the DNA band and detected by a chemiluminescent detection kit (Thermo Fisher). Image Quant LAS 4000 mini was used to observe the membrane.

2.8. Construction of AmpR mutant

The *AmpR* mutant was constructed by the method of Datsenko and Wanner [30]. Following the protocols, primers pair AmpR-forward-1 and AmpR-reverse-2 were designed containing homologous flanking sites of *AmpR* and kanamycin resistance gene (Table 1). The AmpR-forward-1 and AmpR-reverse-2 primers were used to amplify the kanamycin resistance gene flanked by FLP recognition sites from plasmid pKD4. The fragment was cloned into temperature-sensitive (above 30 °C) lambda red recombinase vector pKD46 and transferred to *C. ferundii* competent cells. Kanamycin resistant *C. ferundii* was cured of pKD46 at 37 °C and the kanamycin resistance gene was removed by temperature-sensitive FLP recombinase plasmid pCP20 at 43 °C. The in-frame deletion of *AmpR* was confirmed by DNA sequencing.

2.9. Hydrogen peroxide sensitivity assay

Hydrogen peroxide (H_2O_2) sensitivity was determined by disc diffusion assay in 5 mM H_2O_2. Briefly, the *C. freundii* wild type, and its ΔAmpR overnight culture were 100 times diluted and grown for 2.5 h in a TSB medium. The culture OD_{600} was measured (OD_{600} = 2) and a 200 μl culture was spread on TSA plates (2×10^9 CFU/ml). A blank disc was impregnated in 5 mM H_2O_2 and placed onto the culture plates and zones of inhibition were measured after 24 h.

2.10. Statistical analysis

All reference genes and protein sequences were downloaded from the NCBI database and analyzed by ESPript 3.0 software. Student's t-test (unpaired) was performed by Graph pad prism 8. The significance level was considered $P < 0.05$.

3. Results

3.1. Prevalence of Citrobacter species

During the pandemic, a total of 3492 COVID-19 hospitalized patients were screened for bacterial coinfection and 26 isolates were identified as *Citrobacter* species by the VITEK-2 system. Among the 26 *Citrobacter* spp., 17 were from males and 9 from females. *C. freundii* was the most abundant species (10 isolates), followed by *C. braakii* (8 isolates), *C. koseri* (5 isolates), and *C. amalonaticus* (3 isolates). The majority of samples were isolated from the lower respiratory tract, upper respiratory tract, and urinary tract infections (Fig. 1). Out of the 26 strains, 11 were MDR of which one was ESBL producer. All 26 cases have comorbidities, with the most common type being pneumonia and urinary tract infection. Among the antibiotics, tazobactam, ampicillin, cefotaxime, gentamicin, tobramycin,

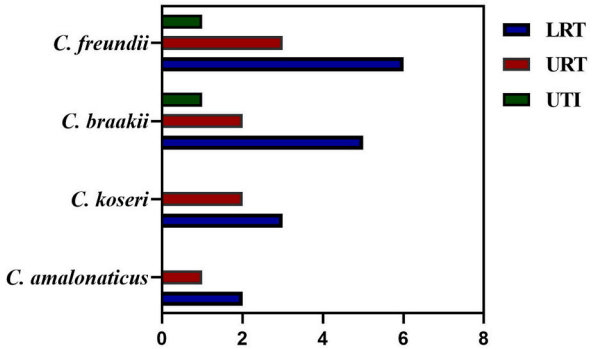

Fig. 1. The prevalence of *Citrobacter* species in COVID-19 patients. The UTI (urinary tract infection), LRT (lower respiratory tract), and LRT (upper respiratory tract) sites were colonized by *Citrobacter* spp., and 26 species were isolated. The bars indicated the number of *C. freundii*, *C. braakii*, *C. koseri*, and *C. amalonaticus* isolated from LRT, URT, and UTI.

and nitrofurantoin were not effective against ESBL strain while sensitive to imipenem and amikacin.

3.2. Characterization of C. freundii ESBL

Initially, the clinical isolates were identified by the VITEK-2 system and followed by API. By API 20E test kit, positive results with ≥89% probability were confirmed as *C. freundii*. The results of *C. freundii* twenty test reactions are provided in Table 2. Further, the genome was extracted and 16 S rRNA sequencing was performed that validated the *Citrobacter* identification by VITEK-2 and API test kit. The *C. freundii* strain displayed resistance to antibiotics such as tazobactam (110 μg), cefotaxime (30 μg), ampicillin (30 μg), gentamicin (10 μg), and nitrofurantoin (110 μg) (Fig. 2A). We performed an ESBL phenotypic assay which revealed that the *C. freundii* strain displayed an 8 mm zone of inhibition on the combination disc (cefotaxime and clavulanic acid) relative to cefotaxime alone that confirmed the production of ESBL by *C. freundii* (Fig. 2B). In the ESBL assay, cefotaxime alone did not kill *C. freundii* while the addition of clavulanic acid inhibited β-lactamases and efficiently kill *C. freundii*. Furthermore, we determined the MIC of the ESBL strain by two-fold broth microdilution method in cation-adjusted MH broth that showed high MICs to tazobactam (256 μg/ml), ampicillin (128 μg/ml), cefotaxime (128 μg/ml), gentamicin (16 μg/ml), tobramycin (16 μg/ml), and ciprofloxacin (≤4 μg/ml). These findings conclude that *C. freundii* produced β-lactamases that inactivated β-lactam antibiotics.

3.3. Identification of mutations in AmpR sequence in C. freundii ESBL strain

Six strains of *Citrobacter* were studied by DNA amplification using primers listed in Table 1, and *AmpC* and *AmpR* genes were detected in *C. freundii* strains. The *AmpC* sequence analysis of the *C. freundii* ESBL strain revealed that the *AmpC* gene was identical to the previously reported chromosome-encoded class C cephalosporinase of the *C. freundii* whereas the *AmpR* gene showed 99% similarity. The ESBL *C. freundii AmpR* gene sequence analysis revealed four point mutations compared to the *C. freundii* reference genome (NCBI accession number NZ_CP033744.1). From the translation of *AmpR* DNA sequences of the ESBL strain, we detected four mutations in the AmpR amino acids sequence namely; Thr64Ile, Arg86Ser, Asp135Val, and Ile183Leu (Fig. 3). Further, we determined the mRNA level of the ESBL strain by RT-qPCR which revealed that the *AmpR* mRNA level was high ($P < 0.05$) relative to the control strain (β-lactam sensitive). We hypothesized that a change in *AmpR* mRNA level might alter the *AmpC* transcript, so we compared the *AmpC* mRNA expression level of the ESBL strain versus the control strain. The results showed that the *AmpC* mRNA level was approximately three fold higher than the control strain and was highly significant ($P < 0.01$) (Fig. 4). We proposed that mutations in *AmpR* increased the expression level of its mRNA that ectopically regulated *AmpC* expression which resulted in the overproduction of AmpC β-lactamase that inactivated the antibiotics. Furthermore, we also analyzed the *AmpD* DNA sequence because previously the *AmpD* mutations were associated with the overproduction of *AmpC* in *E. cloacae* [31]. From the genomic sequence of ESBL strain, we did not detect any change in the DNA sequence of *AmpD* that further confirmed the AmpC β-lactamase high production by *AmpR* mutations. Thus, we conclude that the genetic changes in the transcriptional regulator *AmpR* affected *AmpC* expression that increased the production of AmpC which caused a reduction in the potency of β-lactam antibiotics. Collectively, we reported chromosomal *AmpR* mutations that increased *AmpC* expression and resulted in an escalation in β-lactam antibiotics resistance in the clinical isolate of *C. freundii*.

3.4. AmpR protein binds to the AmpC promoter

The *AmpR* is a transcriptional regulator of the LysR family and contains a helix-turn-helix domain. The promoter site of *AmpC* was

Table 2
The API 20E test results of ESBL *C. freundii* strain.

S. No.	Test (Enzyme/Substrate)	Negative reaction	Positive reaction	*C. freundii* results
1.	O-nitrophenyl-beta-D-galactopyranoside (ONPG)	Colorless	Yellow	+
2.	Arginine dihydrolase (ADH)	Yellow	Red or orange	+
3.	Lysine decarboxylase (LDC)	Yellow	Red or orange	–
4.	Ornithine decarboxylase (ODC)	Yellow	Red or orange	–
5.	Citrate utilization (CIT)	Pale to green	Blue green	+
6.	Hydrogen sulfide (H_2S)	Colorless	Black deposits	+
7.	Urease (URE)	Yellow	Red or orange	–
8.	Tryptophan deaminase (TDA)	Pale yellow or brownish	Brown red	_
9.	Indole (IND)	Pale yellow or colorless	Red (within 2-mints)	_
10.	Voges–Proskauer (VP)	Pale yellow or colorless	Red or pink (within 10-mints)	_
11.	Gelatinase (GEL)	No black diffusion	Black diffusion	–
12.	Glucose (GLU)	Blue or blue-green	Red or yellow	+
13.	Mannitol (MAN)	Blue or blue-green	Red or yellow	+
14.	Inositol (INO)	Blue	Yellow	–
15.	Sorbitol (SOR)	Blue green	Yellow	+
16.	Rhamnose (RHA)	Blue	Yellow	+
17.	Sucrose fermentation (SAC)	Blue	Yellow	+
18.	Melibiose (MEL)	Blue green	Yellow	+
19.	Amygdalin (AMY)	Blue	Yellow	–
20.	Arabinose (ARA)	Blue	Yellow	+

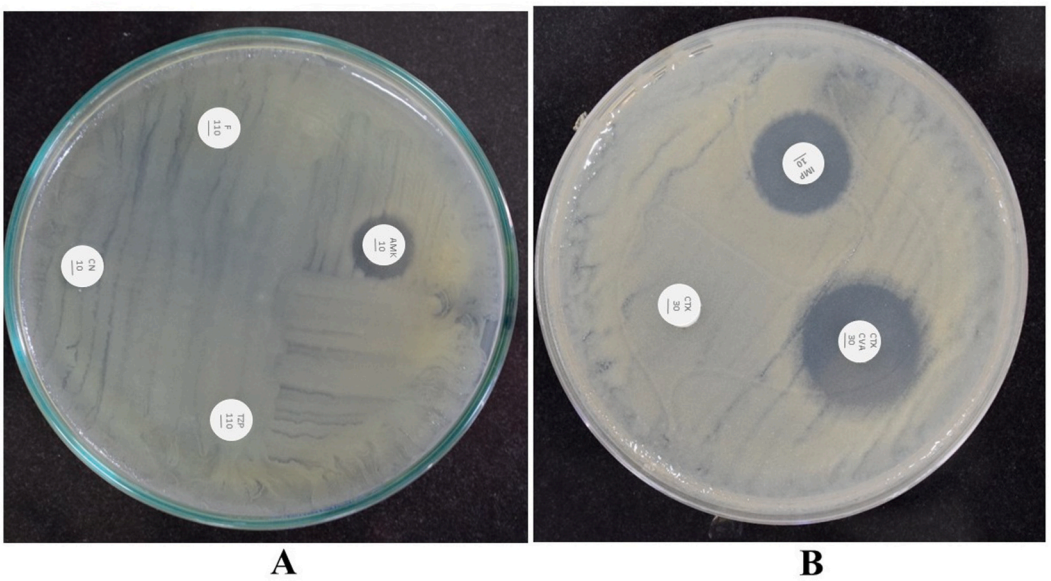

Fig. 2. (A) Antibiotic disc diffusion assay revealed that *C. freundii* is resistant to gentamicin (CN10), nitrofurantoin (F110), and tazobactam (TZP110) while sensitive to amikacin (AMK10) with a zone of inhibition size of 2 mm (B) The ESBL phenotypic assay showed ESBL production and *C. freundii* was resistant to cefotaxime (CFX30) while sensitive to cefotaxime plus clavulanic acid (CTX and CVA30) with a zone of inhibition size of 8 mm. *C. freundii* was sensitive to imipenem (IMP10) with a zone of inhibition size of 5 mm.

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Citrobacter-freundii-AmpR-ESBL-strain     1 MTRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKALEQQLNCQLFVRGSRGL
Citrobacter-freundii-AmpR                 1 MTRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKALEQQLNCQLFVRGSRGL
Citrobacter-braakii-AmpR                  1 MTRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKALEQQLNCQLFVRGSRGL
Citrobacter-werkmanii-AmpR                1 MTRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKALEQQLTCQLFVRGSRGL
Citrobacter-europaeus-AmpR                1 MTRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKALEQQLNCQLFVRGSRGL
consensus>70                                MTRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKALEQQLnCQLFVRGSRGL

Citrobacter-freundii-AmpR-ESBL-strain    61 MLTTEGESLLPVLNDSFDRMAGMLDSFATKQTQEKLKIGVVGTFAVGCLFPLLGDFKRSY
Citrobacter-freundii-AmpR                61 MLTTEGESLLPVLNDSFDRMAGMLDRFATKQTQEKLKIGVVGTFAVGCLFPLLGDFKRSY
Citrobacter-braakii-AmpR                 61 MLTTEGESLLPVLNDSFDRMAGMLDRFATKQTQEKLKIGVVGTFAVGCLFPLLSDFKRHX
Citrobacter-werkmanii-AmpR               61 MLTTEGESLLPVLNDSFDRMAGMLDRFATKQTQEKLKIGVVGTFALGCLFPLLSDFKRSY
Citrobacter-europaeus-AmpR               61 MLTTEGESLLPVLNDSFDRMAGMLDRFATKQTQEKLKIGVVGTFAVGCLFPLLSDFKRNC
consensus>70                                MLTtEGESLLPVLNDSFDRMAGMLDrFATKQTQEKLKIGVVGTFA!GCLFPLL.DFKR..

Citrobacter-freundii-AmpR-ESBL-strain   121 PHIDLHISTHNNRVVPAAEGLDYTIRYGGGAWHDTDAQYLCSALMSPLCSPTLASQIQTP
Citrobacter-freundii-AmpR               121 PHIDLHISTHNNRVDPAAEGLDYTIRYGGGAWHDTDAQYLCSALMSPLCSPTLASQIQTP
Citrobacter-braakii-AmpR                121 PHIDLHISTHNNRVDPAAEGLDYTIRYGGGAWHDTDAQYLCSALMSPLCSPTLASQIKAP
Citrobacter-werkmanii-AmpR              121 PHIDLHISTHNNRVDPAAEGLDYTIRYGGGAWHDTDAQYLCSAPMSPLCSPPLAAQIKVP
Citrobacter-europaeus-AmpR              121 PHIDLHISTHNNRVDPAAEGLDYTIRYGGGAWHDTDAQYLCSAPMSPLCSPTLASQIKAQ
consensus>70                                PHIDLHISTHNNRVdPAAEGLDYTIRYGGGAWHDTDAQYLCSA.MSPLCSPtLAsQI..p

Citrobacter-freundii-AmpR-ESBL-strain   181 ADTLKFPLLRSYRRDEWTLWMQAAGEAPPSPTHNVVVFDSSVTMLEAAQAGIGVAIAPVR
Citrobacter-freundii-AmpR               181 ADTLKFPLLRSYRRDEWTLWMQAAGEAPPSPTHNVVVFDSSVTMLEAAQAGIGVAIAPVR
Citrobacter-braakii-AmpR                181 VDTLKFPLLRSYRRDEWTLWMQAAGEAPPSPTHNVVVFDSSVSMLEAAQAGIGVAIAPVR
Citrobacter-werkmanii-AmpR              181 ADTLKFPLLRSYRRDEWTLWMQAAGEAPPSPTHNVVVFDSSVTMLEAAQAGIGVAIAPVR
Citrobacter-europaeus-AmpR              181 ADTLKFPLLRSYRRDEWTLWMQAAGEAPPSPTHNVVVFDSSVTMLEAAQAGIGVAIAPVR
consensus>70                                aD.LKFPLLRSYRRDEWTLWMQAAGEAPPSPTHNVVVFDSSVtMLEAAQAGIGVAIAPVR

Citrobacter-freundii-AmpR-ESBL-strain   241 MFTHLLSRERIVQPFLTQIDLGSYWITRLQSRPETPAMREFSRWLTGMLHK
Citrobacter-freundii-AmpR               241 MFTHLLSRERIVQPFLTQIDLGSYWITRLQSRPETPAMREFSRWLTGMLHK
Citrobacter-braakii-AmpR                241 MFTHLLSSERIVQPFLTQIDLGSYWITRLQSRPETXAMREFSRWLTGMLH.
Citrobacter-werkmanii-AmpR              241 MFTHLLGSERIVQPFLTQIDLGSYWITRLQSRPETSAMREFSRWLTGVLHQ
Citrobacter-europaeus-AmpR              241 MFRHLLGSERIVQPFLTQIDLGSYWMTRLQSRPETLAMREFSRWLTGVLHQ
consensus>70                                MFtHLL..ERIVQPFLTQIDLGSYWiTRLQSRPET.AMREFSRWLTG.LH.
```

Fig. 3. The AmpR amino acid sequence of *C. freundii* ESBL strain was aligned with *C. freundii*, *C. braakii*, *C. werkmanii*, and *C. europaeus* from the NCBI database and mutations of isoleucine, serine, valine, and leucine were detected in comparison with *C. freundii* reference genome (NCBI accession number NZ_CP033744.1).

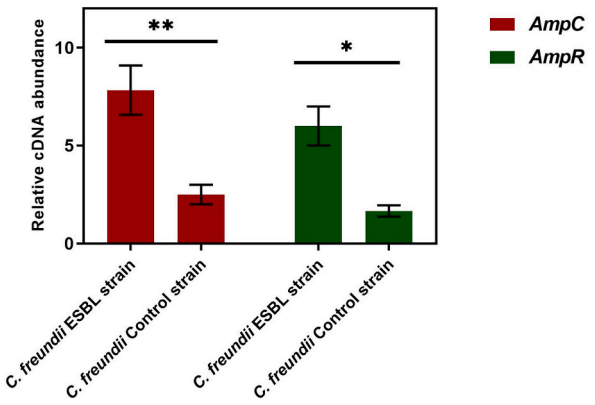

Fig. 4. The RT-qPCR analysis showed high expression of *AmpC* and *AmpR* mRNA levels in the ESBL *C. freundii* strain relative to the control *C. freundii* strain (β-lactam sensitive). The asterisks represent the significance level of mRNA. ✱ $P < 0.05$, ✱✱ $P < 0.01$.

predicted by Softberry BPROM that lay in the intergenic region between AmpR and AmpC. The intergenic region of *AmpR* and *AmpC* consists of 134 bp, and a 60bp DNA probe containing −10 and −35 regions was selected and protein-DNA docking was performed. The protein-DNA probe docking revealed that AmpR has a high docking score (−419.60) than AmpC (−191.13) which indicated a strong binding ability. By analyzing AmpR and AmpC sequence, a helix-turn-helix domain was detected in AmpR which showed binding affinity with the probe (Fig. 5). To validate the docking analysis, the 60bp probe was biotin labeled and protein binding affinity was tested by EMSA. The AmpR protein specifically binds to the 60 bp probe (lane 2) while the AmpC protein (lane 1) cannot bind to the probe which confirmed the docking results (Fig. 6, for uncropped image see Supplementary Fig. S1). The *FrdD* gene lies close to AmpR and was used as a control in EMSA which did not bind to the probe as well (lane 3). From these findings, we conclude that the *AmpR* binds to the *AmpC* promoter and regulates *AmpC* expression in *C. freundii*.

3.5. AmpR regulates C. freundii oxidative stress response

To discern the *AmpR* role in oxidative stress, we performed the H_2O_2 sensitivity assay between wild type and ΔAmpR. The *C. freundii* ΔAmpR was sensitive to H_2O_2 and showed a zone of 11.5 mm (Fig. 7A) whereas the wild type showed a zone of 10 mm (Fig. 7B). The ΔAmpR cell growth was more inhibited than the wild type by H_2O_2 which indicated reduced resistance to H_2O_2. These finding supported the fact that *AmpR* positively regulated oxidative stress response in *C. freundii* and protect the cells during a hostile environment.

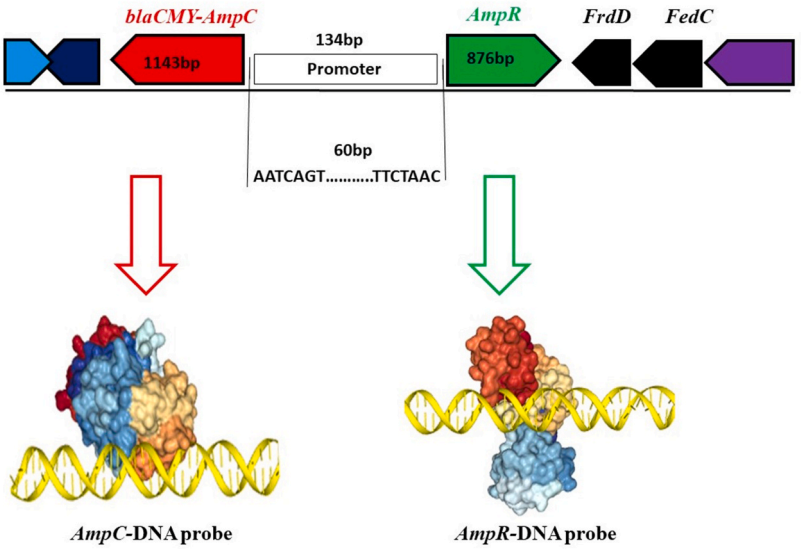

Fig. 5. Docking of protein and DNA probe. The AmpR and AmpC proteins and probe (60bp) were evaluated for possible superimposition via HDOCK (http://hdock.phys.hust.edu.cn/). The AmpR helix-turn-helix domain showed a strong binding affinity with the probe while the AmpC protein did not show any specific binding to promoter.

Probe (fmol)	3	3	3	3
AmpC protein (pmol)	8	0	0	0
AmpR protein (pmol)	0	8	0	0
FrdD protein (pmol)	0	0	8	0
Lysis buffer (µl)	1	1	1	9

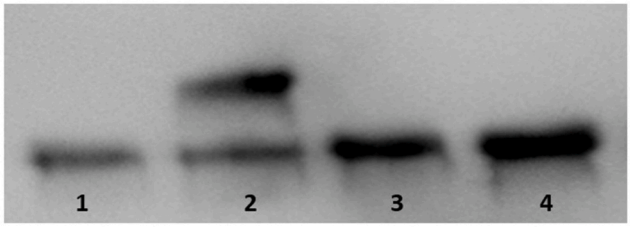

Fig. 6. EMSA results. The shift was observed in lane 2 between the AmpR protein and biotin labeled probe (60bp). Lane 1 represents AmpC protein plus probe; lane 2 AmpR protein plus probe; lane 3 FrdD protein plus probe; and lane 4 probe and lysis buffer. The uncropped and extended figure is provided as a Supplementary Material Fig. S1.

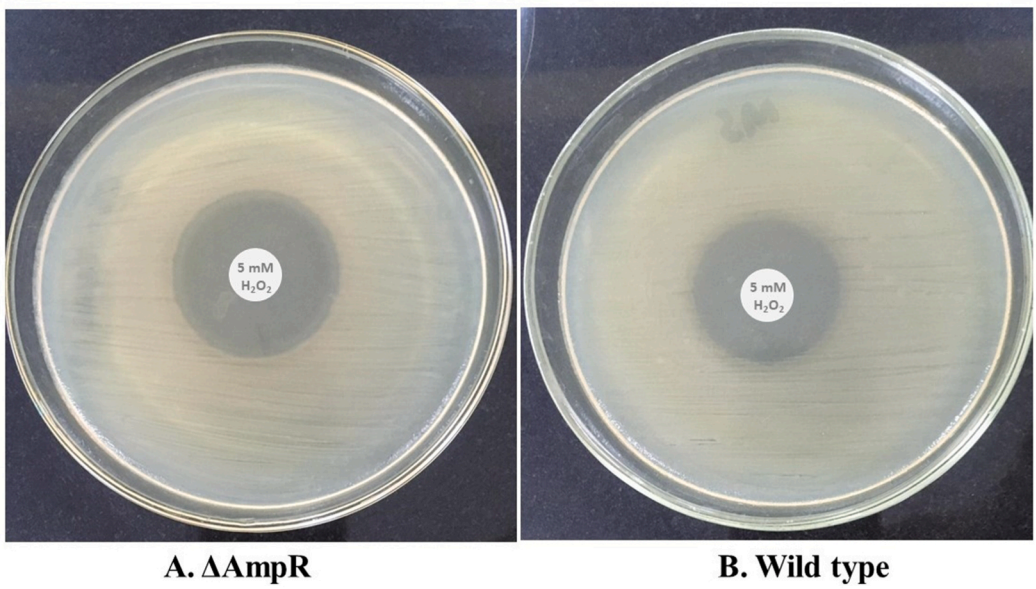

Fig. 7. Hydrogen peroxide sensitivity assay. (A) The H_2O_2 assay revealed that the *AmpR* mutant has a greater zone of inhibition than the wild type (B). The ΔAmpR showed 11.5 mm whereas the wild type showed a 10 mm zone of inhibition.

3.6. AmpR mutant decreased antibiotics resistance

AmpR is required for β-lactam resistance through *AmpC* expression. The mutations in *AmpR* resulted in high expression of *AmpC* that governed β-lactams resistance. We constructed *AmpR* mutant strain that revealed a reduction in β-lactam resistance. The wild type *C. freundii* was resistant to tazobactam (110 µg), cefotaxime (30 µg), ampicillin (30 µg), penicillin (110 µg), gentamicin (10 µg), tobramycin (10 µg), and nitrofurantoin (110 µg) whereas *AmpR* mutant showed a decrease in resistance to tazobactam, gentamicin, tobramycin, nitrofurantoin, and cefotaxime (Fig. 8A and B). The *AmpR* mutant was highly sensitive to cefotaxime, nitrofurantoin, gentamicin, tobramycin, ampicillin, and tazobactam and showed a zone of inhibition size of 11.5 mm, 10 mm, 9 mm, 9 mm, 9.5 mm, and 8 mm respectively whilst no zone was observed in ciprofloxacin (Fig. 8A and B).

4. Discussion

Citrobacter species are typically found in the intestinal and respiratory tracts of humans. *Citrobacter* infections may occur as sporadic or nosocomial outbreaks and are transmitted vertically as well as horizontally [32]. Nosocomial and community acquired *Citrobacter* infections are widely reported [33] and approximately 3–6% of all *Enterobacteriaceae* causing nosocomial infections accounted for *Citrobacter* [34,35]. *Citrobacter* has caused infections worldwide and various reports have concluded urinary and respiratory tracts as predominant sites of infections [36]. Our data reported *Citrobacter* coinfections during the COVID-19 pandemic and found the

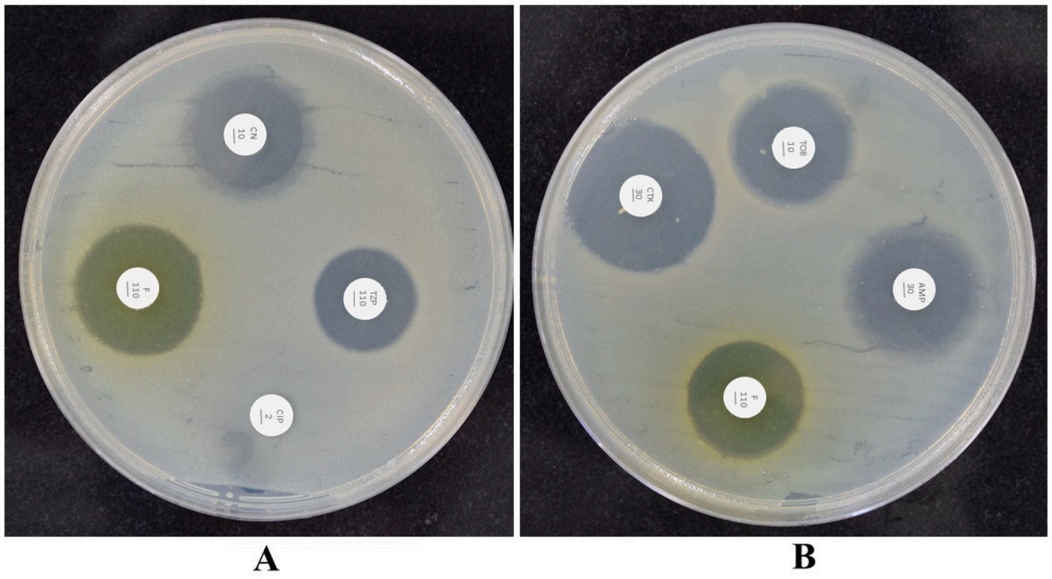

Fig. 8. Antibiotic disc diffusion assay. (A and B) The ΔAmpR mutant was sensitive to gentamicin (CN10), tazobactam (TZP110), ampicillin (AMP30), nitrofurantoin (F110), cefotaxime (CTX10), and tobramycin (TOB10) whereas resistant to ciprofloxacin (CIP2).

respiratory tract as the largely colonized site by *Citrobacter* species. So far, *Citrobacter* species have been isolated from surgical wounds, burns, skin pustules, sputum, urinary tract, lower respiratory tract, and perirectal abscesses [34,36,37]. In the present report, we detected *C. freundii*, *C. braakii*, *C. koseri*, and *C. amalonaticus* in the respiratory tract and urinary tracts, and found predominance in debilitated and elderly patients that suffered from COVID-19. In India, nosocomial, urinary tract, and neonatal septicemia were correlated with *Citrobacter* infections [38] which supported our results of *Citrobacter* coinfections in hospitalized patients. Earlier, *Citrobacter* bacteremia was detected in patients with malignancy, diabetes mellitus, hepatobiliary stones, and heart disease [39] and some studies reported *C. koseri* as the most prevalent species that caused gastrointestinal and urinary tract infections [36,39]. However, our data suggested *C. freundii* as the predominant species which caused infection in COVID-19 patients. Previously, *C. freundii* displayed resistance to tazobactam, gentamicin, cefazolin, ampicillin, norfloxacin, and other antibiotics in hospitalized patients [21, 40], and our data also reported the prevalence of MDR in *C. freundii*. The reason might be the widespread misuse of antibiotics that resulted in the emergence of MDR strains. In countries such as China and India, MDR *C. freundii* was isolated that was sensitive to imipenem, amikacin, and third-generation cephalosporins [41,42]. These results are in accordance with our findings where *C. freundii* showed susceptibility to imipenem and amikacin. Importantly, we detected MDR *C. freundii* which was an ESBL producer that had mutations in the *AmpR* gene which resulted in *AmpC* high expression. Previously, *AmpR* mutations (Arg86Ser) increased the *AmpC* expression in *E. cloacae* [43] and plasmid-encoded *AmpC* (blaCFE-1) also showed *AmpR*-dependent constitutive expression of *AmpC* [44]. In addition, *AmpC* derepression was associated with defects in *the AmpD* gene in *C. freundii* [45], and decreased *AmpD* expression led to *AmpC* overproduction as well as increased β-lactam MICs in clinical isolates of *E. coli*, *C. freundii*, and *P. aeruginosa* [46]. Collectively, their results demonstrated that *AmpD* and *AmpR* modulated the expression of *AmpC* in several bacteria and conferred β-lactam resistance. Our findings conclude that the mutations in *AmpR* modulated the mRNA level of *AmpC* that leads to the production of β-lactamases and inactivation of β-lactam antibiotics. To date, neither *C. freundii* nor other bacteria have been reported with these *AmpR* mutations which are linked with high expression of *AmpC*, resistance to β-lactam, and aminoglycosides. Largely, the β-lactam resistance locus AmpR-AmpC is studied in *P. aeruginosa* and *C. ferundii* that consists of *AmpR* and *AmpC* genes that are divergently transcribed from promoters located in the intergenic region [47]. Generally, *AmpC* expression is controlled by *AmpR* in the presence or absence of inducers and is widely known as the transcriptional regulator of *AmpC* [17]. The AmpR-AmpC locus is highly conserved in *Enterobacteriaceae*, for instance, in *E. coli* and *Shigella sonnei*, β-lactams resistance is mediated by tandem duplications of the *AmpC* gene and promoter mutations located within the upstream of fumarate reductase operon [48,49] while in *C. freundii*, *E. cloacae*, and *S. marcescens*, *AmpC* expression is induced by β-lactam antibiotics that produce β-lactamases which inactivate β-lactam antibiotics [50, 51]. Currently, we have shown that AmpR protein regulates *AmpC* expression by specifically binding to a 60bp fragment of *AmpC* promoter whilst AmpC could not bind. These results are in accordance with the previous findings where the AmpR helix turn helix motif was involved in DNA binding and autoregulation [18]. Similar results were also reported in *P. aeruginosa*, where in silico study revealed an AT-rich intergenic region of AmpR-AmpC as a putative *AmpR* binding site [52]. Besides, the *AmpR* deletion resulted in low expression of *AmpC* and a major decrease in β-lactam and aminoglycosides resistance in *C. ferundii* whereas similar results were only reported in *P. aeruginosa* [10,47]. In addition, the *AmpR* mutant showed increased sensitivity to H_2O_2 which confirmed *AmpR* role in protection against oxidative stress response. To date, there is no such report that delineated the involvement of *AmpR* in oxidative stress responses. Altogether, our results showed the isolation of ESBL *C. freundii* from the hospitalized patient and revealed *AmpR*

mediated *AmpC* high expression and ΔAmpR changes in *C. ferundii* that are associated with a decrease in β-lactam and aminoglycosides resistance. The present study has several limitations. First, it did not characterize all *Citrobacter* species isolated and only focus on ESBL *C. freundii*. Secondly, all the *Citrobacter* species were isolated from COVID-19 patients and the rest of the hospitalized patients were not included. The current study highlighted the prevalence of *Citrobacter* coinfection in hospitalized patients but did not report the clinical data of patients. Lastly, it remains possible that certain *Citrobacter* strains were re-isolated via regular patient transfers or through the readmission of previously discharged patients during the study period.

5. Conclusion and future prospective

In summary, we have detected mutations in *AmpR* (Thr64Ile, Arg86Ser, Asp135Val, and Ile183Leu) that conferred resistance to β-lactam antibiotics in *C. freundii*. The ΔAmpR decreased β-lactam and aminoglycosides resistance and suppressed *AmpC* expression. The tetra mutant strain was an ESBL producer that reflected a changing pattern of drug resistance in *C. freundii* which altered the local susceptibility profile of the *Citrobacter* species in Pakistan. The data presented in this report illustrated the spectrum of *Citrobacter* infection in COVID-19 patients during the pandemic and revealed *C. freundii* is one of the commonest causes of coinfection in hospitalized patients. Knowingly, β-lactamase inhibitors have made a great impact in hospitals as adjunct therapies, however, the emergence of ESBL made clinical outcomes more complicated and the emergence of MDR strains. Antibiotic resistance breakers such as ESBL inhibitors represent a promising avenue of research for ESBL-producing *Citrobacter* species. Researchers need to explore the genetic determinants that bacteria adopt during resistance development, such as mutation and acquiring resistance genes. Besides, the control of antibiotic resistance is associated with proper antibiotic prescription, physician vigilance, and more proactive strategies to maximize the lifespans of current antibiotics through antimicrobial stewardship and public awareness.

Author contribution statement

Falak Naz Tariq: Mehreen Shafiq: Nadeem Khawar: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. GUL HABIB: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper. Haji Gul: Performed the experiments; Wrote the paper. Azam Hayat: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Mujaddad Ur Rehman: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data. Ihab Mohamed Moussa: Eman A. Mahmoud: Hosam O. Elansary: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Data availability statement

No data was used for the research described in the article.

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Ethics approval

The study was approved by the Department of Microbiology, Abbottabad University of Science and Technology (AUST/ORIC/2021/375).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19486.

References

[1] R. Vivas, A.A.T. Barbosa, S.S. Dolabela, S. Jain, Multidrug-resistant bacteria and alternative methods to control them: an overview, Microb. Drug Resist. 25 (6) (2019) 890–908.
[2] K. Bush, Bench-to-bedside review: the role of β-lactamases in antibiotic-resistant Gram-negative infections, Crit. Care 14 (3) (2010) 1–8.
[3] G. De Angelis, P. Del Giacomo, B. Posteraro, M. Sanguinetti, M. Tumbarello, Molecular mechanisms, epidemiology, and clinical importance of β-lactam resistance in Enterobacteriaceae, Int. J. Mol. Sci. 21 (14) (2020) 5090.
[4] H. Gul, G. Habib, I.M. Khan, S.U. Rahman, N.M. Khan, Y. Liu, Genetic resilience in chickens against bacterial, viral and Protozoal pathogens, Front. Vet. Sci. (2022) 1776.
[5] A. Smith, L. Saiman, J. Zhou, P. Della-Latta, H. Jia, P.L. Graham III, Concordance of gastrointestinal tract colonization and subsequent bloodstream infections with Gram-negative bacilli in very low birthweight infants in the neonatal intensive care unit, Pediatr. Infect. Dis. J. 29 (9) (2010) 831.
[6] R.P. Ambler, The structure of β-lactamases, Philos. Trans. R. Soc. Lond. B Biol. Sci. 289 (1036) (1980) 321–331.
[7] K. Bush, G.A. Jacoby, A.A. Medeiros, A functional classification scheme for beta-lactamases and its correlation with molecular structure, Antimicrob. Agents Chemother. 39 (6) (1995) 1211–1233.
[8] K. Bush, G.A. Jacoby, Updated functional classification of β-lactamases, Antimicrob. Agents Chemother. 54 (3) (2010) 969–976.
[9] M. Barlow, B.G. Hall, Origin and evolution of the AmpC β-lactamases of Citrobacter freundii, Antimicrob. Agents Chemother. 46 (5) (2002) 1190–1198.
[10] K.-F. Kong, S.R. Jayawardena, S.D. Indulkar, A. Del Puerto, C.-L. Koh, N. Høiby, K. Mathee, Pseudomonas aeruginosa AmpR is a global transcriptional factor that regulates expression of AmpC and PoxB β-lactamases, proteases, quorum sensing, and other virulence factors, Antimicrob. Agents Chemother. 49 (11) (2005) 4567–4575.
[11] E. Bartowsky, S. Normark, Purification and mutant analysis of Citrobacter freundii AmpR, the regulator for chromosomal AmpC β-lactamase, Mol. Microbiol. 5 (7) (1991) 1715–1725.
[12] R. Nakano, R. Okamoto, N. Nagano, M. Inoue, Resistance to gram-negative organisms due to high-level expression of plasmid-encoded ampC β-lactamase blaCMY-4 promoted by insertion sequence ISEcp1, J. Infect. Chemother. 13 (1) (2007) 18–23.
[13] M.D. Reisbig, N.D. Hanson, The ACT-1 plasmid-encoded AmpC β-lactamase is inducible: detection in a complex β-lactamase background, J. Antimicrob. Chemother. 49 (3) (2002) 557–560.
[14] P. Giakkoupi, A. Tambic-Andrasevic, S. Vourli, J. Skrlin, S. Sestan-Crnek, L. Tzouvelekis, A. Vatopoulos, Transferable DHA-1 cephalosporinase in Escherichia coli, Int. J. Antimicrob. Agents 27 (1) (2006) 77–80.
[15] V. Miriagou, L. Tzouvelekis, L. Villa, E. Lebessi, A. Vatopoulos, A. Carattoli, E. Tzelepi, CMY-13, a novel inducible cephalosporinase encoded by an Escherichia coli plasmid, Antimicrob. Agents Chemother. 48 (8) (2004) 3172–3174.
[16] J. Park, Why does Escherichia coli recycle its cell wall peptides? Mol. Microbiol. 17 (3) (1995) 421–426.
[17] F. Lindberg, L. Westman, S. Normark, Regulatory components in Citrobacter freundii ampC beta-lactamase induction, Proc. Natl. Acad. Sci. USA 82 (14) (1985) 4620–4624.
[18] S. Lindquist, F. Lindberg, S. Normark, Binding of the Citrobacter freundii AmpR regulator to a single DNA site provides both autoregulation and activation of the inducible ampC beta-lactamase gene, J. Bacteriol. 171 (7) (1989) 3746–3753.
[19] C. Jacobs, L. Huang, E. Bartowsky, S. Normark, J. Park, Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction, EMBO J. 13 (19) (1994) 4684–4694.
[20] R. Nakano, A. Nakano, H. Yano, R. Okamoto, Role of AmpR in the high expression of the plasmid-encoded AmpC β-lactamase CFE-1, mSphere 2 (2017) 4, https://doi.org/10.1128/msphere.00192-00117.
[21] L. Liu, D. Chen, L. Liu, R. Lan, S. Hao, W. Jin, H. Sun, Y. Wang, Y. Liang, J. Xu, Genetic diversity, multidrug resistance, and virulence of Citrobacter freundii from diarrheal patients and healthy individuals, Front. Cell. Infect. Microbiol. 8 (2018) 233.
[22] G. Habib, K. Mahmood, H. Gul, M. Tariq, Q.U. Ain, A. Hayat, M.U. Rehman, Pathophysiology of methicillin-resistant Staphylococcus aureus superinfection in COVID-19 patients, Pathophysiology 29 (3) (2022) 405–413.
[23] G. Habib, M.S.Z. Khan, H. Gul, A. Hayat, M.U. Rehman, A Persistent High Ambient Temperature Waned the Community Spread of Severe Acute Respiratory Syndrome Coronavirus-2 in Pakistan, Elsevier, City, 2022.
[24] G. Habib, K. Mahmood, L. Ahmad, H. Gul, A. Hayat, M.U. Rehman, Clinical manifestations of active tuberculosis patients coinfected with severe acute respiratory syndrome coronavirus-2, Journal of Clinical Tuberculosis and Other Mycobacterial Diseases 31 (2023), 100359.
[25] T.K. Ling, P. Tam, Z. Liu, A.F. Cheng, Evaluation of VITEK 2 rapid identification and susceptibility testing system against gram-negative clinical isolates, J. Clin. Microbiol. 39 (8) (2001) 2964–2966.
[26] M. Wikler, Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard, tenth ed., Clinical and laboratory standards institute., 2009.
[27] G. Habib, J. Zhu, B. Sun, A novel type I toxin-antitoxin system modulates persister cell formation in Staphylococcus aureus, International Journal of Medical Microbiology 310 (2) (2020), 151400.
[28] G. Habib, Q. Zhu, B. Sun, Bioinformatics and functional assessment of toxin-antitoxin systems in Staphylococcus aureus, Toxins 10 (11) (2018) 473.
[29] V. Jarlier, M.-H. Nicolas, G. Fournier, A. Philippon, Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns, Clin. Infect. Dis. 10 (4) (1988) 867–878.
[30] K.A. Datsenko, B.L. Wanner, One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products, Proc. Natl. Acad. Sci. USA 97 (12) (2000) 6640–6645.
[31] K. Kaneko, R. Okamoto, R. Nakano, S. Kawakami, M. Inoue, Gene mutations responsible for overexpression of AmpC β-lactamase in some clinical isolates of Enterobacter cloacae, J. Clin. Microbiol. 43 (6) (2005) 2955–2958.
[32] T.I. Doran, The role of Citrobacter in clinical disease of children, Clin. Infect. Dis. 28 (2) (1999) 384–394.
[33] R.N. Jones, S.G. Jenkins, D.J. Hoban, M.A. Pfaller, R. Ramphal, In vitro efficacy of six cephalosporins tested against Enterobacteriaceae isolated at 38 North American medical centres participating in the SENTRY Antimicrobial Surveillance Program, 1997–1998, Int. J. Antimicrob. Agents 15 (2) (2000) 111–118.
[34] B.A. Lipsky, E.W. Hook III, A.A. Smith, J.J. Plorde, Citrobacter infections in humans: experience at the Seattle Veterans Administration Medical Center and a review of the literature, Rev. Infect. Dis. 2 (5) (1980) 746–760.
[35] J.-P. Lavigne, C. Defez, N. Bouziges, A. Mahamat, A. Sotto, Clinical and molecular epidemiology of multidrug-resistant Citrobacter spp. infections in a French university hospital, Eur. J. Clin. Microbiol. Infect. Dis. 26 (6) (2007) 439–441.
[36] K. Ranjan, N. Citrobacter Ranjan, An emerging health care associated urinary pathogen, Urol. Ann. 5 (4) (2013) 313.
[37] S. Arens, J. Verhaegen, L. Verbist, Differentiation and susceptibility of Citrobacter isolates from patients in a university hospital, Clin. Microbiol. Infection 3 (1) (1997) 53–57.
[38] P. Panigrahi, D.S. Chandel, N.I. Hansen, N. Sharma, S. Kandefer, S. Parida, R. Satpathy, L. Pradhan, A. Mohapatra, S.S. Mohapatra, Neonatal sepsis in rural India: timing, microbiology and antibiotic resistance in a population-based prospective study in the community setting, J. Perinatol. 37 (8) (2017) 911–921.
[39] C.-C. Shih, Y.-C. Chen, S.-C. Chang, K.-T. Luh, W.-C. Hsieh, Bacteremia due to Citrobacter species: significance of primary intraabdominal infection, Clin. Infect. Dis. 23 (3) (1996) 543–549.
[40] L. Liu, L. Qin, S. Hao, R. Lan, B. Xu, Y. Guo, R. Jiang, H. Sun, X. Chen, X. Lv, Lineage, antimicrobial resistance and virulence of Citrobacter spp, Pathogens 9 (3) (2020) 195.
[41] H. Sami, A. Sultan, M. Rizvi, F. Khan, S. Ahmad, I. Shukla, H.M. Khan, Citrobacter as a uropathogen, its prevalence and antibiotics susceptibility pattern, CHRISMED J. Health Res. 4 (1) (2017) 23.
[42] L. Liu, R. Lan, L. Liu, Y. Wang, Y. Zhang, Y. Wang, J. Xu, Antimicrobial resistance and cytotoxicity of Citrobacter spp. in maanshan anhui province, China, Front. Microbiol. 8 (2017) 1357.

[43] A. Kuga, R. Okamoto, M. Inoue, ampR gene mutations that greatly increase class C β-lactamase activity in Enterobacter cloacae, Antimicrob. Agents Chemother. 44 (3) (2000) 561–567.
[44] R. Nakano, R. Okamoto, Y. Nakano, K. Kaneko, N. Okitsu, Y. Hosaka, M. Inoue, CFE-1, a novel plasmid-encoded AmpC β-lactamase with an ampR gene originating from Citrobacter freundii, Antimicrob. Agents Chemother. 48 (4) (2004) 1151–1158.
[45] P. Stapleton, K. Shannon, I. Phillips, DNA sequence differences of ampD mutants of Citrobacter freundii, Antimicrob. Agents Chemother. 39 (11) (1995) 2494–2498.
[46] A.J. Schmidtke, N.D. Hanson, Model system to evaluate the effect of ampD mutations on AmpC-mediated β-lactam resistance, Antimicrob. Agents Chemother. 50 (6) (2006) 2030–2037.
[47] D. Balasubramanian, H. Kumari, K. Mathee, Pseudomonas aeruginosa AmpR: an acute–chronic switch regulator, Pathogens disease 73 (2) (2015) 1.
[48] S.T. Cole, J.R. Guest, Production of a soluble form of fumarate reductase by multiple gene duplication in Escherichia coli K12, Eur. J. Biochem. 102 (1) (1979) 65–71.
[49] B. Jaurin, T. Grundström, S. Normark, Sequence elements determining ampC promoter strength in E. coli, EMBO J. 1 (7) (1982) 875–881.
[50] S. Cole, M. Nicolas, Beta-lactam resistance mechanisms in gram-negative bacteria, Microbiol. Sci. 3 (11) (1986) 334–339.
[51] F. Lindberg, S. Normark, Common mechanism of ampC beta-lactamase induction in enterobacteria: regulation of the cloned Enterobacter cloacae P99 beta-lactamase gene, J. Bacteriol. 169 (2) (1987) 758–763.
[52] D. Balasubramanian, L. Schneper, M. Merighi, R. Smith, G. Narasimhan, S. Lory, K. Mathee, The regulatory repertoire of Pseudomonas aeruginosa AmpC ß-lactamase regulator AmpR includes virulence genes, PLoS One 7 (3) (2012), e34067.