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Detection of multidrug resistance in Enterobacteriaceae in drinking water†

Visakha Singh and M. Kiranmai Reddy *

The rise of antibiotic resistance (AMR) and multidrug resistance (MDR) in the pathogenic Enterobacteriaceae family has become a major global concern in recent years. Waterborne infections still account for a significant mortality burden worldwide, especially within marginalised communities. Resistance, particularly in the case of *Citrobacter* species, has come into focus owing to their association with various nosocomial infections, UTIs, and opportunistic infections in immunocompromised patients. Herein, a study was conducted in marginalised areas during the monsoon season, involving the collection and analysis of drinking water samples from five different sites. The samples were analysed for enteric bacteria, among which *Citrobacter coronae* Tue2_1 was identified. This isolate was identified based on biochemical testing and genetic analysis involving 16S rDNA sequencing. The isolate was found to showcase MDR against beta-lactams, such as ampicillin and amoxiclav; macrolides such as azithromycin; and nitrofurantoin, a widely used nitrofuran. Intermediate resistance was also discovered against meropenem. This study identified resistance in a less-studied *Citrobacter* strain, highlighting the need to thoroughly monitor drinking water sources for their role as reservoirs of antimicrobial resistance.

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Environmental significance

Our study reveals a crucial public health concern: the presence of multidrug-resistant *Citrobacter coronae* Tue 2_1 in drinking water in low-income communities dependent on municipal/groundwater supplies. Despite access to safe water being a fundamental right, vulnerable populations face disproportionate exposure to this pathogen, which is resistant to ampicillin and azithromycin and shows emerging meropenem resistance. This contamination creates a dangerous cycle, whereby the compromised water quality in marginalized areas breeds antibiotic-resistant infections that are increasingly difficult to treat. The findings of this study emphasize the urgent need for equitable water quality monitoring, improved sanitation infrastructure, and comprehensive AMR surveillance in environmental sources. Without immediate intervention, these communities risk being trapped in a health crisis where basic infections become untreatable, aggravating existing socioeconomic inequalities. This situation demands a coordinated response addressing water safety and antimicrobial management to protect our most vulnerable populations.

1. Introduction

Drinking water is one of the most precious resources necessary for maintaining life. Its necessity for a healthy life has been aptly justified by the inclusion of access to safe drinking water as a basic human right by the General Assembly of the United Nations in 2010.[1] Additionally, the Sustainable Development Goals (SDGs) include universal access to safe drinking water as a target for countries to achieve by 2030.[2] Despite this, numerous issues are still faced by individuals across the globe regarding access to safe drinking water free from microbial contaminants such as Enterobacteriales.[3,4] One such bacterial species has been identified as *Citrobacter* spp. This species has been studied for its roles in hospital-acquired (nosocomial) infections and urinary tract infections (UTIs) and its association with opportunistic infections in immunocompromised individuals. While *Citrobacter* species can be found in the environment, they can inhabit the human gastrointestinal tract. *Citrobacter cronae* was discovered in Germany in rectal swabs of patients suffering from diarrhoea in 2020.[5] *Citrobacter* species are increasingly being reported as being resistant to various antibiotics, especially beta-lactams, such as ampicillin, which is in part owing to their ability to acquire resistance *via* horizontal genetic transfer from different bacterial species.[6,7] Resistance to carbapenems, such as meropenem, has also been reported in various countries, including India and China, and has developed, as in the case of *Citrobacter*, by the transfer of genes.[8,9] In terms of drinking water, numerous studies have reported the presence of *Citrobacter* species, as well as instances of antimicrobial-resistant (AMR) and multidrug-resistant (MDR) species.[10–12] Nitrofurantoin is a nitrofuran antibiotic that has been regularly used and has proven to be effective against UTIs

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throughout its wide-scale usage for many decades, albeit it has been reported to be notably ineffective against some cases of *Citrobacter* species. Several studies have reported that certain bacterial species exhibit a low tendency to develop resistance against nitrofurantoin, likely due to the complex mechanisms required for resistance acquisition.[13–15] Isolating Enterobacteriaceae from drinking water is a global concern since it hints at the possible lack of effectiveness of water-treatment procedures. This could indicate increasing resistance among the bacterial population in the water source against treatment agents such as chlorine and the need for improved procedures and training to avoid system failures.[16] The scope of the current study included the investigation of AMR and MDR in *Citrobacter* extracted from drinking water in low-income urban households in Visakhapatnam City, India. It aims to fill crucial gaps in understanding the prevalence of AMR in drinking water sources and its potential health risks to marginalised communities. The study isolated and characterized *Citrobacter* species, including MDR strains, in drinking water in urban settings, which represents an area less explored than hospital settings.

2. Experimental section

2.1 Sample collection

Sampling was conducted in Arilova, a low-income neighbourhood within the urban limits of Visakhapatnam, India (latitude 17.766483, longitude 83.3161317). This location was chosen due to its limited water resources and the resident's primary dependence on the municipality's water supply and groundwater sources. Water samples were collected from five different sources: three from municipality water-treatment plants (raw water, chlorinated water, and overhead tank), and two from groundwater (hand pumps) and residential water taps running treated municipality water. These samples were collected in triplicate sterile polypropylene bottles and placed immediately in an icebox and kept there until they arrived at the laboratory for analysis. The collection was performed during the monsoon season (July to October) in 2024 in Visakhapatnam. A total of 30 samples were collected from the study area during the research.

2.2 Microbial analysis

The collected water samples were tested for the presence of bacterial isolates using selective and differential agar media, such as xylose lysine deoxycholate (XLD) agar, *Salmonella* and *Shigella* (SS) agar, and deoxycholate citrate agar (DCA).[17] The agar plates were inoculated using the spread plate method and incubated for 24 to 48 h at 37 °C in an incubator. The isolated colonies were streaked onto the agar media to procure pure cultures for further analysis. Gram staining was conducted to visualise the pure cultures under a microscope.[18] Biochemical tests, comprising IMViC tests, starch hydrolysis tests, hydrogen sulfide production tests, and nitrate reduction tests, were carried out for identification of the isolates.[19] Biochemical test kits were also employed for analysis of the isolates. An antimicrobial susceptibility test was conducted on the colonies of bacterial isolates to determine resistance using the Kirby–Bauer method.[20] All the agar, antibiotics, reagents, and kits used for our research were procured from HiMedia Laboratories, India.

2.3 Genetic analysis

The isolated pure culture was identified using 16S rDNA gene sequencing, followed by the use of the NCBI BLAST nucleotide tool. DNA isolated from the culture was first evaluated for its quality using a 1.0% agarose gel, during which a high molecular weight, single band of DNA was observed. Amplification was performed on a fragment of 16S rDNA using the 27F and 1492R primers. An amplicon of 1500 bp was observed on agarose gel, which was then purified for forward and reverse DNA sequencing. This sequencing was performed with forward and reverse primers using a BDT v3.1 cycle kit on an ABI 3730xl Genetic Analyser. Aligner software generated consensus sequences of the 16S rDNA gene using the forward and reverse sequencing data. This sequence was then analysed using the NCBI BLAST nucleotide tool, and the results were produced using the GenBank database[21] (Nucleotide BLAST),[22] as presented in Fig. 1.S.† During the analysis, a Megablast search was performed on a database of 16s ribosomal RNA sequences with the scoring parameters set to the linear gap costs and the match/mismatch scores at (1,−2). The first 10 sequences were selected and aligned using the multiple alignment software program Clustal W. With the help of MEGA 7, a distance matrix and phylogenetic tree were also constructed for the isolate.[23] The strain's evolutionary history was surmised using the maximum likelihood method based on the Kimura 2 parameter model.[24] A bootstrap consensus tree was developed from 1000 replicates, with branches corresponding to parts reproducing less than 50% of the bootstrap replicates collapsed to prepare a phylogenetic tree, as presented in Fig. 1 and 2. The percentage of replicate trees that showed the clustering of associated taxa during the bootstrap test was mentioned beside the tree branches (Felsenstein, 1985).[25] Initially, the trees were obtained using the Neighbour-Join and BioNJ algorithms to give a pairwise distances matrix estimated by the maximum composite likelihood (MCL) approach. A topology with a superior log likelihood value was then chosen. This analysis used 11 nucleotide sequences and eliminated all positions with gaps and

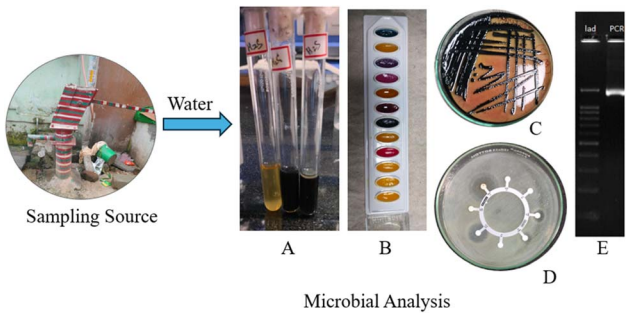

Fig. 1 (A) Voges–Proskauer test. (B) Biochemical test kit. (C) Isolated culture on DCA. (D) Antibiotic resistance test using Mueller–Hinton agar with antibiotic discs. Resistance shown for meropenem (MRP), azithromycin (AZM), and nitrofurantoin (NIT). (E) 16S PCR Amplicon.

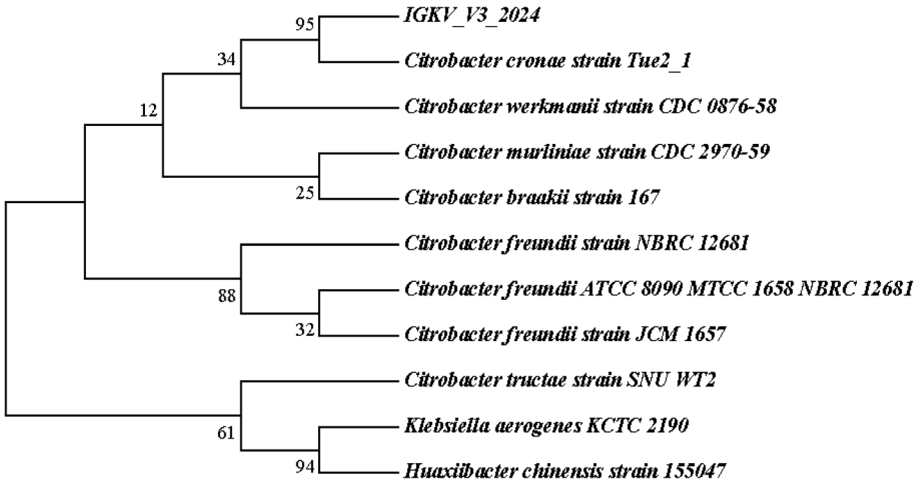

Fig. 2 Phylogenetic analysis of the isolated strain sequence using the maximum likelihood method performed in MEGA 7.

missing data. A total of 1456 positions were present in the final dataset. The sequence was submitted in the NCBI GenBank database with the accession number PV546871.[26]

3. Results

3.1 Microbial analysis

The isolated species were found to be Gram-negative bacilli, grown on XLD and DCA agar. The bacterial culture was observed as black colonies in the agar media. Biochemical tests were performed on the isolated bacteria, with the results presented in Table 1. The isolation was favourable for hydrogen sulfide production and could utilise citrate, glucose, arabinose, sorbitol, and lactose. The isolated colonies were tested for antibiotic resistance using antibiotic discs on Mueller–Hinton agar. This isolate was found to be resistant to azithromycin (AZM), which is a macrolide, nitrofurantoin (NIT), a nitrofuran antibiotic, beta-lactams, ampicillin (AMP), and amoxiclav (AMC). Intermediate resistance was also shown against meropenem (MRP), a carbapenem-type of antibiotic (Table 2). The microbial analysis results are depicted in Fig. 1.

Table 1 Results of the biochemical tests

S. no.	Biochemical test	Result
1	Methyl red (MR)	+
2	Voges–Proskauer (VP)	−
3	Indole	−
4	Nitrate reduction	+
5	Citrate utilisation	+
6	Starch hydrolysis	−
7	Lysine utilisation	−
8	Ornithine utilisation	+
9	Urease	+
10	Phenylalanine deaminase	−
11	Hydrogen sulfide production	+
12	Glucose	+
13	Adonitol	−
14	Lactose	+
15	Arabinose	+
16	Sorbitol	+

Table 2 Identification of IGKV_V3_2024 interactions with antibiotics

S. no.	Antibiotics	Resistance	Intermediate resistance	Susceptibility
1	Meropenem	0%	11%	89%
2	Nitrofurantoin	33%	0	67%
3	Ampicillin	25%	0	75%
4	Azithromycin	21%	0	79%
5	Amoxiclav	21%	0	79%

3.2 Genetic analysis

The isolated sample (I) was found to show maximum similarity with *Citrobacter cronae* Tue2_1, based on nucleotide homology and phylogenetic analysis. It belongs to the family of Enterobacteriaceae and order Enterobacteriales. The genetic analysis results are depicted in Fig. 1(E) and 2, showcasing the agarose gel electrophoresis and phylogenetic analysis results, respectively.

4. Discussion

In our study, the isolate IGKV_V3_2024 was identified as *Citrobacter cronae* through genetic analysis. This isolate was found to grow on media like XLD, SS, and DCA agars, which are generally utilised for isolating and identifying enteric bacterial species, such as *Salmonella* and *Shigella*. Through biochemical analysis, we ascertained the profile of this isolate, which was also correlated with its physical appearance as black-coloured colonies on the selective agar, owing to the production of hydrogen sulfide. The isolated species showed multidrug resistance, being resistant to antibiotic drugs, such as

nitrofurantoin, and beta-lactams, such as ampicillin and amoxiclav. According to ref. 13, the use of nitrofurantoin in a judicious manner was found to be favourable for treating urinary tract infections due to its durability against several factors contributing to resistance. While many previous studies have indicated NIT resistance is limited, it has started emerging in newer studies conducted on pathogenic bacterial species.[27,28] It has also been noted that resistance to NIT could indicate extensive drug resistance among Enterobacteriaceae.[14] This resistance has been associated with mutations in the nfsA and nfsB genes responsible for processing nitrofurans, and accumulating toxic intermediates within the bacterial cells, thereby resulting in cell death.[29] These mutations were found to cause the synthesis of inactive nitroreductases, enzymes that catalyse the reduction of nitro compounds, including nitrofurans. Moreover, mutations in the ribE gene were also noticed to aid in resistance. Horizontal gene transfers can help different bacterial species acquire oqxAB gene, coding for a multidrug efflux pump. A combination of some or all of these factors could render robust resistance to Enterobacteriaceae against NIT.[30] It was also observed that the incidence of multiple factors, such as a large deletion mutation of these genes, would be required unless intrinsic resistance was developed in the organism.[13] Due to the requirement for the inactivation of multiple factors to gain resistance against NIT, it might become necessary for an organism exhibiting low-level resistance to populate and establish well within an area.[31] Hence, studies have called for strict vigilance during the administration of this drug to limit the spread of resistant variants.[28]

In one study,[10] *Citrobacter* species were isolated from drinking water wells in Zanzibar, which revealed their potential for antimicrobial resistance. In another study,[32] multiple *Citrobacter* species were isolated from diarrhoea patients in China, showing multidrug resistance towards antibiotics like meropenem and amikacin. While numerous studies have been performed on antimicrobial resistance in *Citrobacter* species in clinical samples, very few studies have been done on environmental samples. However, in one study conducted on the water of the Narmada River,[33] environments such as freshwater resources were presented as potential harbours for MDR *Citrobacter* species. Meanwhile, our study was performed on isolated *Citrobacter cronae*, which was initially discovered in Germany from the rectal swabs or stool samples of patients.[5] This strain has been studied comparatively less, especially regarding MDR species isolated from environmental samples. There is clearly a need for further studies into the prevalence of resistance in bacterial species, especially *Citrobacter* spp., to understand the scope of MDR trends in clinical and environmental settings.

5. Conclusion

Drinking water has been recognised as a universal human right, highlighting its value as essential to living a healthy life. Safe and easily accessible drinking water is also a significant benchmark for achieving the UN SDGs for all nations. In this regard, MDR bacterial isolates, such as *Citrobacter cronae*, can cause concern. While this strain has not been well studied, *Citrobacter* spp. has been associated with nosocomial infections, UTIs, and opportunistic infections in immunocompromised individuals. In the present study, the isolate showcased resistance towards beta-lactams, such as ampicillin, and nitrofurantoin, a commonly used antibiotic for treating UTIs. The isolation of bacterial species, such as *Citrobacter cronae*, from environmental and drinking water sources needs to be further studied to understand their relevance towards the AMR and MDR phenomena.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

There are no conflicts to declare.

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