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ANTIBIOTIC RESISTANCE OF *CITROBACTER SPP.* ISOLATED FROM LAKES OF UDAIPUR, INDIA

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ABSTRACT

Citrobacter is an opportunistic pathogen that can cause diarrhea, urinary tract infection, respiratory tract infection and acute meningitis. In health care settings *Citrobacter* species have become an increasing cause of concern as they are multi drug resistant (MDR) and associated with high mortality between 30-60%. In the present study an attempt has been made to isolate *Citrobacter spp.* from lakes of Udaipur namely Fateh sagar and Pichhola and to test their antibiotic resistance against 15 commonly used antibiotics i.e. gentamycin, kanamycin, tetracycline, erythromycin, ampicillin, penicillin, polymyxin B, amikacin, ciprofloxacin, vancomycin, rifampicin, chloremphenicol, streptomycin, cefixime, trimethoprim by disc diffusion method. A total of 12 strains were isolated, tentatively identified and grouped into 2 species as *Citrobacter freundii and Citrobacter braakii* according to their biochemical characteristics. Genomic DNA of these isolates was extracted and amplified with universal primers 27 F and 1492 R. The amplified products were subjected to sequencing to confirm their identification. All the strains which were identified as *Citrobacter freundii* were found resistant to 7 antibiotics namely ampicillin, penicillin, vancomycin, kanamycin, polymyxin B, rifampicin, and streptomycin. All the strains which were identified as *Citrobacter braakii* were found resistant to 8 antibiotics namely ampicillin, penicillin, vancomycin, kanamycin, polymyxin B, rifampicin, erythromycin and streptomycin, out of 15 antibiotics used in the study. The results indicated that the water of both the lakes was contaminated with multi-antibiotic resistant enteric pathogenic bacteria. This study thus provides valuable information

for making policy decisions aimed at reducing microbial contamination of lake water and the indiscriminate use of antibiotics.

KEYWORDS: *Citrobacter braakii*, *Citrobacter freundii*, Fateh Sagar, Pichhola, lake, Udaipur.

1. INTRODUCTION

Gram negative bacteria belonging to *Enterobacteriaceae* family, which is critically important as a matter of human health, are prevalent in nature and foods. They especially exist in water and materials contaminated by excrement. Consequently, they are important sources of infection for humans when they consume contaminated foods. Infections caused by bacteria resistant to antibiotics significantly cause mortality and economical losses. Trimethoprimsulfamethoxazol antibiotics and beta lactam inhibitors are frequently used in the treatment of *Citrobacter*, *Proteus* and *Providencia*, which belong to the family *Enterobacteriaceae*. (Uraz et al., 2009).

Citrobacter spp. is commonly found in freshwater systems. *Citrobacter* is a gram negative, facultative anaerobe which causes diarrhea, urinary tract infection, respiratory tract infection and acute meningitis in humans and is also pathogenic for aquatic and terrestrial animals.

Resistant bacteria are becoming commonplace in healthcare institutions. Bacterial resistance often results in treatment failure, which can have serious consequences, especially in critically ill patients. The surfacing of antibiotic resistance usually results from the misuse of antibiotics as growth-promoters in animal production, for therapy and prophylaxis (Barbosa et al., 2000). Because humans consume these animal products, there is a probability of the spread of resistant strains from animals to humans and thus healthy individuals can become carrier hosts for multiple antibiotic-resistant bacteria (Reinthaler et al., 2003). Nutrient-rich environments like sewage and wastewater create optimal conditions to promote horizontal gene transfer processes (Igbinosa and Okoh, 2012). Studies have documented the detection of antimicrobial resistance in wastewater and drinking water (Schwartz, 2003). Therefore, ubiquitous bacteria, which are capable of colonizing different water types, are of particular interest to assess potential forms of antimicrobial resistance dissemination. The genus *Citrobacter* comprises ubiquitous bacteria, considered indigenous to aquatic environments (Janda and Abbott, 2010). Given their ubiquity in water environment and patterns of acquired antimicrobial resistance, members of the genus *Citrobacter* are good examples of such

bacteria. Therefore in the present study an attempt has been made to detect the antibiotic resistance in the *Citrobacter spp.* isolated from water of lakes of Udaipur.

2. MATERIAL AND METHODS

Isolation and Biochemical characteriazation

Bacterial strains were isolated from water samples of lake Fateh Sagar and lake Pichhola on nutrient agar medium after an incubation at 37^{o}C for 24h. Isolated strains were selected according to their morphological and cultural characteristics. Selected isolates were subjected to biochemical characterization. The tests include gram staining, motility, catalase test, oxidase test, citrate utilization, urea hydrolysis, methyl red test, voges-proskauer test, indole production, nitrate reduction, growth on MacConkey agar and carbohydrate fermentation. These results will be further matched to the criteria provided in Bergey's Manual of Systematic Bacteriology.

Molecular characterization

DNA Isolation

The genomic DNA from bacterial cultures of tentatively identified *Citrobacter* strains grown overnight in nutrient broth, was extracted according to the method given by Pospiech and Neumann (1995).

Primers

For DNA sequencing bacterial 16S rRNA gene based universal primers namely 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'CGGTTACCTTGTTACGACTT-3') designed by Weisburg *et al.* (1991) were used.

PCR amplification

The reaction mixture (20µl) contained 10 pmol of each primer,0.2 mM of each dNTP ($MgCl_2$), 1X PCR buffer, 2µl of DNA solution and 1 U/µl of Taq DNA polymerase (Banglore genei). Amplification was carried out in a thermal cycler as follows: initial denaturation at 95 °C for 5 min, then 35 cycles consisting of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min, and a final extension for 10 min. at 72°C. Final hold of amplified PCR products was at 4°C. Amplified products were visualized on a 2.0% agarose gel along with 500bp DNA ladders. The PCR products amplified with universal primers were submitted to Bangalore Genei Pvt. Ltd., Bangalore, India for sequencing. The sequences of the 16S rRNA of the isolates were compared with

available standard sequences of bacterial lineages in the NCBI Genebank using nBLAST. The obtained sequences were submitted to the NCBI Genebank.

Antibiotic susceptibility testing

Antibiotic susceptibility test of *Citrobacter* strains was determined according to the disc-diffusion method of Kirby- Bauer (Bauer *et al.*, 1966) on Mueller-Hinton agar plates. A total of fifteen antibiotics namely ampicillin (AMP 10µg/disc), amikacin (AK 30 µg/disc), cefixime (CFM 5 µg/disc), ciprofloxacin (CIP 5 µg/disc), chloremphenicol (C 30 µg/disc), erythromycin (E 15 µg/disc), gentamicin (GEN 30 µg/disc), kanamycin (K 30 µg/disc), penicillin (P 10 µg/disc), polymyxin (PB 300 µg/disc), rifampicin (RIF 30 µg/disc), streptomycin (S 25 µg/disc), tetracycline (TE 30 µg/disc), trimethoprim (TR 5 µg/disc) and vancomycin (VA 30 µg/disc) were used. Results were interpreted according to the guidelines of the CLSI (CLSI, 1999).

3. RESULTS AND DISCUSSION

A total of 116 isolates (56 from lake Fateh Sagar and 60 from lake Pichhola) were isolated on nutrient agar medium after an incubation at 37°C for 24h. Out of them 12 isolates were preliminary characterized as *Citrobacter Spp.* on the basis of morphological, cultural and biochemical characterization. The results for the same are presented in **Table 1**.

All these 12 isolates were found gram negative, motile and rod shaped and showed small/ medium in size, circular form, moist, entire margin, flat/convex elevation. All the isolates were found positive for catalase test, citrate utilization, MR test and nitrate reduction. All these 12 isolates were found negative for oxidase test, VP test, urea hydrolysis, gelatin hydrolysis, starch hydrolysis, Indole production and casein hydrolysis. Out of these 12 strains, a total of 7 strains (FS 7, FM 30, FM 36, PS 74, PM 91, PM 101 and PW 115) were found to be able to produce H_2S and hydrolyse arginine. All the 12 isolates were found able to grow on MacConkey agar. All the 12 isolates were found to be able to ferment lactose, maltose, dextrose, fructose, rhamnose, cellobiose and mannitol. Out of these 12 strains, a total of 7 strains (FS 7, FM 30, FM 36, PS 74, PM 91, PM 101 and PW 115) were found to be able to ferment sucrose and arabinose and remaining 5 isolates (FS 13, FM 32, FW 53, PS 70 and PM 80) were not found to be able to ferment sucrose and arabinose. All the 12 strains were not found to be able to ferment raffinose. These results were further matched with Bergey's Manual of Systematic Bacteriology. A total of 7 strains (FS 7, FM 30, FM 36, PS 74, PS 71, PM 91, PM 101 and PW 115) were tentatively identified as *Citrobacter freundii*

and remaining 5 isolates (FS 13, FM 32, FW 53, PS 70 and PM 80) were tentatively identified as *Citrobacter braakii.*

Table 1: Biochemical characteristics of bacterial isolates.

Biochemical characteristics	***Citrobacter freundii* (n=7)**	***Citrobacter braakii* (n=5)**
Gram's reaction	-	-
shape	rod	rod
Motility at 37°C	+	+
Catalase activity	+	+
Oxidase activity	-	-
Indole production	-	-
MR reaction	+	+
VP reaction	-	-
Citrate utilization	+	+
Nitrate reduction	+	+
H2S production	+	-
Arginine hydrolysis	+	-
Urea hydrolysis	-	-
Starch hydrolysis	-	-
Casein hydrolysis	-	-
Gelatin liquification	-	-
Growth on MacConkey agar	+	+
Carbohydrate fermentation		
Lactose	+	+
Maltose	+	+
Dextrose	+	+
Sucrose	+	-
Raffinose	-	-
Rhamnose	+	+
Arabinose	+	-
Cellobiose	+	+
Fructose	+	-
Mannitol	+	-

For molecular identification, PCR amplification of DNA isolated from all these 12 strains was done using universal primers 27F and 1492R. The amplified products gave 1500 bp product on 2% agarose gel **(Fig. 1)**. On the basis of the sequence similarity of the partial 16S rRNA sequences of all the strains compared with available standard sequences of bacterial lineages in NCBI Genebank reference strains; 7 isolates, 3 from lake Fateh Sagar FS 7, FM 30, FM 36 and 4 isolates from lake Pichhola PS 74, PM 91, PM 101 and PW 115 were identified as *Citrobacter freundii.* A total of 5 isolates 3 from lake Fateh Sagar FS 13, FM 32,

FW 53 and 2 from lake Pichhola PS 70 and PM 80 were identified were identified as *Citrobacter braakii.*

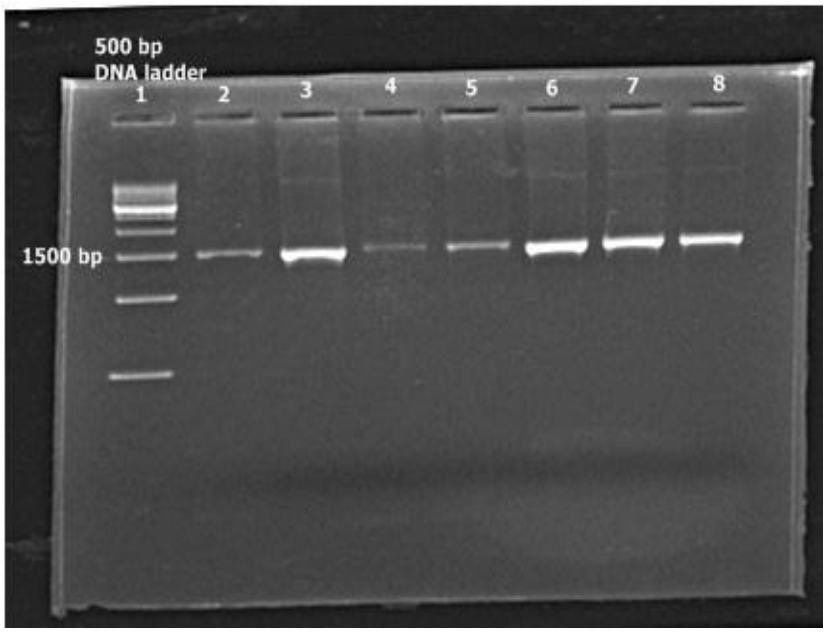

Fig 1: Identification of isolates by using bacterial 16S rRNA specific universal primer 27F and 1492R in PCR, Lane 1- 500bp ladder, lane 2 standard *Citrobacter freundii,* lane 3-8 *Citrobacter freundii* FS 7, *Citrobacter freundii* PS 74, *Citrobacter freundii* PM 91, *Citrobacter braakii* FS 13, *Citrobacter braakii* PS 70 and *Citrobacter braakii* PM 80.

The nucleotide sequences of all these strains were deposited in NCBI gene bank under the allotted accession numbers **(Table 2).**

Table 2: Molecular identification of isolates using 16S rRNA sequencing.

S. No.	Name of isolate	Identification	% Similarity	Ascession No.
1.	FS 7	*Citrobacter freundii*	99	KP635373
2.	FM 30	*Citrobacter freundii*	99	KP739917
3.	FM 36	*Citrobacter freundii*	99	KP739919
4.	PS 74	*Citrobacter freundii*	100	KR149314
5.	PM 91	*Citrobacter freundii*	99	KR149315
6.	PM 101	*Citrobacter freundii*	99	KR149316
7.	PW 115	*Citrobacter freundii*	99	KR149317
8.	FS 13	*Citrobacter braakii*	100	KP739918
9.	FM 32	*Citrobacter braakii*	99	KP739920
10.	FW 53	*Citrobacter braakii*	100	KP739921
11.	PS 70	*Citrobacter braakii*	100	KP969055
12.	PM 80	*Citrobacter braakii*	100	KJ410764

The antibiotic resistance of *Citrobacter freundii* and *C. braakii* isolated and identified in the present study showed a full range of resistance (0-100%) for the 15 antibiotics **(Table 3)** which are commonly used in humans and in aquaculture. The presence of *Citrobacter spp*. in drinking water is undesirable, as may have implications for user health, mainly via contact transmission (WHO, 2008). Nevertheless, they have been detected in different types of drinking water, namely tap, mineral bottled and wells (Biscardi *et al.*, 2002; Pablos *et al.*, 2009).

Table 3: Antibiotic resistance among *Citrobacter freundii* and *Citrobacter braakii* isolated from lake Fateh Sagar and lake Pichhola of Udaipur.

S. No.	Antibiotics	% of resistant strains	
		Citrobacter freundii **(n=7)**	***Citrobacter braakii*** **(n=5)**
1.	ampicillin (AMP 10µg/disc)	100	100
2.	amikacin (AK 30 µg/disc)	0	20
3.	cefixime (CFM 5 µg/disc),	0	0
4.	ciprofloxacin (CIP 5 µg/disc)	0	0
5.	chloremphenicol (C 30 µg/disc	0	0
6.	erythromycin (E 15 µg/disc),	28.57	80
7.	gentamicin (GEN 30 µg/disc)	0	0
8.	kanamycin (K 30 µg/disc)	71.42	100
9.	penicillin (P 10 µg/disc)	100	100
10.	polymyxin (PB 300 µg/disc)	71.42	80
11.	rifampicin (RIF 30 µg/disc)	57.14	80
12.	streptomycin (S 25 µg/disc)	57.14	80
13.	tetracycline (TE 30 µg/disc)	0	0
14.	trimethoprim (TR 5 µg/disc)	0	0
15.	vancomycin (VA 30 µg/disc)	100	100

In many countries, the release of pathogenic bacteria in faeces dispersed into the aquatic environment can contaminate these waters. Once these bacteria are in the aquatic environment, plasmid exchange between the bacteria is readily facilitated and can result in a higher frequency of multiple antibiotic resistant strains (Hatha *et al.*, 2005).

The misuse of antimicrobial agents leads to the high incidences of multi drug resistance and development of resistant strains from drug sensitive microorganisms from antibiotic saturated environment (Jacob, 2007). All the strains which were tentatively identified as *Citrobacter freundii* were found resistant to 7 antibiotics namely ampicillin (100%), penicillin (100%), vancomycin (100%), kanamycin (71.42%), polymyxin B (71.42%), rifampicin (57.14%), and streptomycin (57.14%). All the strains which were tentatively identified as *Citrobacter*

braakii were found strongly resistant to 8 antibiotics ampicillin (100%), penicillin (100%), vancomycin (100%), kanamycin (100%), rifampicin (80%), polymyxin B (80%) erythromycin (80%) and streptomycin (80%) out of 15 antibiotics used in the study. All the 12 isolates showed sensitivity to amikacin, ciprofloxacin, chloremphenicol, tetracycline, gentamicin, cefixime and trimethoprim. Uraz et al. (2009) also isolated trimethoprim sensitive *Citrobacter spp.* from miscellaneous samples similar to our study. Poonia et al. (2014) also determined antibiotic succeptibility profile of citrobacter and other bacteria isolated from natural sources of water and rural areas of Sikkim. They found that isolates showed high levels of antibiotic resistance to ampicillin, whereas less resistance to amikacin and gentamicin.

Mandal et al. (2011) isolated multidrug-resistant *Citrobacter freundii* which showed high resistance to ampicillin and less resistance to gentamicin and amikacin similar to the results of present study.

CONCLUSION

The effectiveness of antibiotic treatment of diseases will decline due to the development of antibiotic resistance in the environment. It is evident from the present results that multiple antibiotic-resistant bacteria can thrive in water that can serve as an environmental reservoir and can therefore provide a route to multidrug-resistant pathogens to enter human population. In this study, high incidence of multiple antibiotic resistance amongst *Citrobacter* species was observed suggesting these lakes as a reservoir of antibiotic resistance determinants in the study communities. The high antibiotic resistance also indicates a negative impact on therapy with these classes of antibiotics. Strict quality control measures should be put in place to ensure proper treatment of lake water. This would ensure the discharge of properly treated wastewater into the lake to prevent the occurrence and spread of water- and food-borne diseases and to remove such pathogens as *Citrobacter* species is here advocated to prevent the dissemination of multidrug resistant determinants into the receiving waterbodies.

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