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RESEARCH ARTICLE

Multiple Antibiotic Resistance profile of environmental Citrobacter isolates harboring virulent markers isolated from fresh water reverine environment of Narmada, India

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Abstract

Infection due to *Citrobacter* spp. is of great concern and indiscriminate uses of commonly used antimicrobial drugs have led to resistance in *Citrobacter* species. The study was aimed to assess the antibiotic profile of *Citrobacter* strains harboring virulent genes. Out of 125 isolates analyzed, 23 isolates were positive for *hly* A gene whereas *via* B gene was found only in all the isolates of *Citrobacter freundii*. Antibiogram analysis of these 51 virulent isolates revealed that *Citrobacter braakii* and *Citrobacter sedlakii* are been found to be more resistant than other isolates of *Citrobacter* spp. In iodometric analysis, beta lactamase activity was observed within one minute in *Citrobacter koseri* and *Citrobacter freundii* isolates. Among 51 strains analyzed, 37 of them harbored multiple plasmids that exhibited a diverse distribution pattern. The study demonstrated the existence of multidrug resistant *Citrobacter* sp. harboring *hly* A and *via* B genes in fresh water of river Narmada. The occurrence of virulent antibiotic resistant *Citrobacter* sp. in riverine environment may contribute significantly to its dissemination and if enters directly or indirectly in human food chain can even cause consternation.

Introduction

Virulence as a concept is intrinsically coupled to disease and is therefore most easily measured in terms of morbidity and mortality. *Citrobacter freundii*, a member of the genus *Citrobacter* within the family Enterobacteriaceae, is classically considered a commensal resident in the intestinal tracts of both humans and animals. It has been shown that some isolates have acquired virulence traits and have caused diarrhea and other infections in humans (Doran, 1992). Studies on the *Citrobacter* species in fresh water have demonstrated its potential risks (Losch et al. 2008) due to production of some virulence factors (Lin et al. 2007; Goska et al. 2011). Overtime, they acquire plasmids, integrons or transposon containing resistance genes and virulence factors that transform these bacteria into more virulent and resistant organism. This possibility must be explored because resistant bacteria are classically less virulent than susceptible one but they remain pathogenic especially in immunocompromised hosts (Vila et al. 2002). This may be due to presence of putative virulent factors that produces number of toxins eg. enterotoxins or hemolysin that causes severe clinical manifestations of infections (Paraje et al. 2005).

Infection due to *Citrobacter* spp. can be community acquired or arise nosocomially. *Citrobacter* have been long the subject of a number of antimicrobial resistance studies (Nada et al. 2004; Lavigne et al. 2007; Mohanty et al. 2007; Thapa et al. 2009). Resistance patterns are generally influenced by year of isolation, classes of antimicrobial agents and pressure exerted by antimicrobials use (Wang et al. 2002). It was noticed over the past decades that *Citrobacter*

strains have progressively become resistant to most widely used antimicrobials viz. ampicillin, ceftazidime, cefotaxime, aminoglycoside and tetracycline (Underwood and Avison, 2004).

Whatever the source of antibiotic resistance or virulence, such traits are apparently found in natural water resources (Ash et al. 2002). Studies on *Citrobacter* are restricted mainly to clinical studies involving urinary tract infections (Nada et al. 2004; Lavigne et al. 2007) or bacteremias and sepsis (Samonis et al. 2009). Moreover, there are no published data revealing the evidence of such resistance strains in river water in India, even none of the report carried virulence factor analysis of *Citrobacter* isolates from riverine environment. River Narmada is the largest west flowing river of peninsular India and harbors a hitherto of Enterobacteriaceae members harboring virulent determinants (Sharma et al. 2005; 2007; 2008; 2009)

Thus the present study was conducted with an objective to assess the antibiotic profile of *Citrobacter* strains isolated from riverine system harboring virulent genes in absence of primate host against commonly prescribed antimicrobial agents for their consternating situation.

Materials and Methods

Bacterial Isolates

Bacterial cultures were obtained from Bacterial Germplasm Collection Center (BGCC), Department of Biological Science, R.D. University Jabalpur, isolated from 11 different stations of river Narmada which were confirmed as *Citrobacter* spp. on the basis of biochemical (Krieg and Holt, 1984; Jenda and Abott, 2006; Bryant 1993) and molecular characteristics using Randomly Amplified Polymorphic DNA analysis (RAPD) (Purohit et al. 2011), Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Selvakumaran et al. 2008), Repetitive Extragenic Palindromic analysis (REP) and Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) analysis (Versalovic et al. 1991; Woods et al. 1992).

PCR Analysis of Virulence Genes

The DNA was isolated by following the method of Ausubul et al. (1995). *hly* A gene was determined by following Heuzenroeder et al. (1999) method. 25 µl reaction mixture was prepared by adding 12.3 µl milli Q water, 2.5 µl of 10x PCR Buffer (with 500 mM KCl and 15 mM $MgCl_2$), 2.0 µl dNTPs (200 mM), 2.5 µl each primer (25 pM each of forward and backward), 0.2 µl *Taq* Polymerase (3 U/µl) and 3 µl template DNA. The amplification protocol consisted of 35 cycles of 0.5 min at 94^{o}C, annealing for 0.5 min at 62^{o}C (hlyA), and 2 min extension at 72^{o}C for 1 min in a Corbett Research Thermal Sequencer, Model FTS-960 (Sydney, Australia). PCR products were separated in 1.5% agarose gels at 64.5 mA for 3 h and visualized under UV light.

A PCR assay for the primers VIAB-1 from bp 5867 to 5888 (TGTCGAGCAGATGGATGAGCAT), and VIAB-2, from bp 6362 to 6383 (ACGGCTGAAGGTTACGGACCGA) were used to detect the presence of Vi antigen responsible for virulence in *Citrobacter* isolates (Lin et al. 2007). 25 µl PCR reaction mixture was prepared by adding 200 µM of each nucleotide i.e. dATP, dGTP, dTTP, and dCTP, 15 pmol of each primer to amplify 516 bp via B fragment, 1.5 mM $MgCl_2$, 1 ng of genomic DNA as template and 0.2 units of *Taq* DNA polymerase (Bangalore Genie, India) and amplified by following incubation at 95^{o}C for 2 min; at 85^{o}C for 5 min, 5 cycles of denaturation at 95^{o}C for 1 min. followed by annealing at 58^{o}C for 1 min. and extension at 75^{o}C for 1 min. The denaturation (95^{o}C, 30 sec), annealing (58^{o}C, 1 min) and extension (75^{o}C, 1 min) steps were repeated for 25 cycles. The reaction mixture was incubated at 62^{o}C for 2 min and at 72^{o}C for 10 min. PCR products were separated in 3% agarose gels containing ethidium bromide (0.5 mg/ml) at 40 volt for 3 h and visualized under ultraviolet light.

Antibiotic Susceptibility Test

Antibiotic susceptibility test was carried out by the standard disk diffusion method as per Clinical and Laboratory Standards Institute (CLSI, 2005) guidelines using commercially available antibiotic disks using Mueller Hinton agar medium (HiMedia, and CDH, India). A standard strain of *C. koseri* (BLCk/002) was used as control. The 18 antibiotics tested were: ampicillin/sulbactum (10/10 µg), carbenicillin B (100 µg), amoxyclave (30 µg), penicillin (2 U), piperacillin/tazobactam (100 µg/10 µg), cefoxitin (30 µg), aztreonam (30 µg), meropenem (10 µg), gentamicin (10 µg), cotrimoxazole (23.75/1.25µg), ciprofloxacin (5 µg), polymixin B (300 U), cefuroxime (30 µg), ceftazidime (30 µg), tobramycin (10 µg), amikacin,(30 µg), cefazolin (30 µg) and streptomycin (25 µg). After incubation at 37°C, organisms were classified as sensitive, intermediate, or resistant. All the experiments were performed in duplicates.

Multiple Antibiotic Resistance (MAR) index

Multiple Antibiotic Resistance (MAR) index of each isolate was calculated as

MAR = y/nx,

where,

y is the actual number of resistance determinants scored

n is the number of isolate tested &,

x is total number of antibiotic tested (Manjusha et al. 2005).

Determination of β-Lactamase Activity by Iodometric Method

0.1 ml solution containing 10,000 U of benzyl penicillin-G/ ml in 0.1 M potassium phosphate buffer (pH 7.0 and pH 8.0) was added in micro tube. Bacterial growth from agar was suspended in this solution and kept at room temperature for 60 min. Thereafter, 20 µl volume of 1% (w/v) soluble starch was added in it. β-lactamase activity was demonstrated by decolorization of iodine within 10 min (Catlin, 1975).

Plasmid Profile

Plasmids were isolated from a 5-ml overnight grown culture in Luria Bertani medium using alkaline lysis method (Ausubel et al. 1995). Plasmid DNA was separated on 1% agarose gel, containing 2 µg/ml of ethidium bromide and observed under UV trans-illuminator. Lambda DNA/Hind III digest (Bangalore Genie, India) were used as known molecular weight marker.

Results

PCR Analysis of Virulence Genes

During study, 125 isolates of *Citrobacter* were investigating using primer pair VIAB-1 and VIAB-2 to determine the presence of *via* B gene fragment. *Via*-B gene was present in only 39 isolates of *C. freundii* while it was absent in other *Citrobacter* sp. Studied (Fig. 1). Out of the 125 strains of *Citrobacter* only 9/39 of *C. freundii*, 7/44 of *C. koseri*, 4/18 of *C. braakii*, 1/7 of *C. sedlakii* and 1/5 of *C. werkmanii* were positive for *hly* A gene (Fig. 2).

Antibiogram Analysis of *Citrobacter* Spp. Isolated From the River Narmada

The antibiotic resistance/susceptibility was tested in only 51 *Citrobacter* isolates which were found to be positive for *hly* A gene and *via* B gene. Out of 39 isolates of *C. freundii*, most of the strains showed resistance against carbenicillin, amoxyclave, cefuroxime, amoxyclave, cefoxitin, piperacillin/tazobactam, polymixin B, ampicillin-sulbactum, penicillin-G, tobramycin and ceftazidime (Table 1). Likewise, out of 7 isolates of *C. koseri*, most of the strains showed resistance against aztreonam, penicillin-G, cefazolin, ceftazidime, polymixin B, cefoxitin, and cefuroxime. Out of 4 isolate of *C. braakii* most of the strains showed resistance against aztreonam, cefuroxime, amoxyclave, cefoxitin, piperacillin/tazobactam, polymixin B, cefazolin, penicillin-G, tobramycin and piperacillin-tazobactam. One isolate of *C. werkmanii* showed resistance against carbenicillin, cefuroxime, amoxyclave, cefoxitin, polymixin B, cefazolin, penicillin G and piperacillin-tazobactam. An isolate of *C. sedlakii* showed resistance against carbenicillin, aztreonam, cefuroxime, amoxyclave, polymixin B, ampicillin-sulbactum, ceftazidime, cefazolin, penicillin G, gentamicin, and piperacillin/ tazobactam (Fig. 3). Multidrug resistance observed in *C. freundii*, *C. koseri*, *C. braakii*, *C. sedlakii* and *C. werkmanii* was 51.6%, 47.58%, 61.11%, 66.66% and 44.44% respectively (Fig. 4). Multiple antibiotic resistance (MAR) index ranged from 0.111 to 0.722 (Table 1).

β - Lactamase Activity

In the present study all the 51 isolates showed β-lactamase activity within 30 sec. to 10 min (Fig. 5). Most promising β-lactamase producer was *Citrobacter koseri* followed by *C. freundii* and *C. braakii*.

Plasmid Profiling

Upon plasmid profiling of 51 isolates, 37 isolates generated 18 different banding patterns. Among these 14 isolates harbored multiple plasmids with an average of 564-23130 bp (Table 2). 94.54% isolates harbored plasmids of 9416 to 23130 bp and 6.10% isolates harbored plasmids of 2000 to 3000 bp.

Discussion

The present study is an effort to critically evaluate virulent factors harboring *Citrobacter* isolates based on antibiogram analysis and plasmid profiling. Antimicrobial resistance in enteric pathogens is of great significance in the developing world, where the rate of diarrheal diseases is highest and it has been observed that the plethora of antibiotic resistant bacteria discharge in fresh water which is increasing progressively (Junco-Diaz et al. 2006). In a study performed at Trinidad (Orrett and Shurland, 2000), among all the gram-negative organisms tested, *Citrobacter* isolates displayed varying degrees of multidrug-resistance while all other isolates showed >80% susceptibility. In another study on drug resistance of urinary pathogens from a tertiary care hospital in South India, *Citrobacter* sp. showed varying degree of resistance to different antibiotics like ampicillin (100%), cefazolin (83.3%), gentamicin (55.6%), nitrofurantion (89.9%) and norfloxacin (77.8%) (Mohanty et al. 2007). The widespread use of broad spectrum antibiotics might be the principle factor in the emergence and dissemination of such high level of antimicrobial resistant strains in fresh water of river Narmada. The present study illustrates the spectrum of *Citrobacter* in fresh water of river Narmada and indicates that *Citrobacter* is most often associated with multidrug resistance. Overall, it could be observed that such antimicrobial resistance pattern (carbenicillin, aztreonam, cefuroxime, amoxyclave, cefoxitin, ceftazidime, cefazolin, tobramycin and penicillin G) was prevalent among *Citrobacter* isolates isolated from clinical samples of patients of India (Nada et al. 2004), Nepal (Thapa et al. 2009), Greece (Samonis et al. 2009) and in a patient returned to France from India (Poirel et al. 2011). This resistance might be due to acquisition of resistance genes like β-lactamase, efflux pumps or alternation in the porins which acts synergistically as a channel for a drug entry to confer resistance among *Citrobacter* isolates (Zhang et al. 2009).

During the study, higher percentage of resistance was observed against β-lactam antibiotic, which may be due to production of β-lactamase. Since, all the isolates were susceptible towards carbapenems; it was assumed that this can be kept as reserve drug for the treatment of infections caused by *Citrobacter* isolates.

In the present investigation, multiple antibiotic resistances (MAR) index ranged from 0.111 to 0.722, indicating higher resistance acquired by the isolates. MAR index higher than 0.2 identifies that organism was originated from high-risk sources of contamination, where antibiotics are in use (Ash et al. 2002). There is a clear evidence that if resistant bacteria enters in fresh water stream, their presence is associated with the risk of transferring resistant genes from harmless bacteria to pathogenic one or to human interacting with aquatic environments (Costanzo et al., 2005).

The β-lactamase enzyme reacts with β-lactam bond and forms acyl enzyme intermediates, which undergoes rapid hydrolysis of β-lactam ring and results in the loss of antibiotic activity (Oliva et al. 2003). According to Sharma et al. (2008) all bacteria produce at least one chromosomally mediated β-lactamase and these enzymes are specific for genus, species, and subspecies. In this study, the *C. freundii* and *C. koseri* showed β-lactamase induction within 30 sec to 1 min. which may be due to the constitutive secretion of β-lactamase enzyme inside the bacterial cell. The positive test indicated that implementation of intervention strategies should be considered for treatment of various ailments caused by penicillin resistant *Citrobacter* sp. During the study, an interesting feature among *Citrobacter* isolate was observed that 87.62% were cefoxitin resistant which may be due to β-lactamase production.

According to Pepperell et al. (2002) plasmid is essential for virulence and its presence differentiates the pathogenic bacteria from non pathogenic one. During the study, 72.54% of *Citrobacter* strains harbored plasmid of variable size. There was also a high degree of plasmids relatedness among the *Citrobacter* isolates because of the presence of similar size plasmids especially of 23130, 9416 and 6557 bp. During the present study, an isolate harboring 3 small plasmids bands showed resistant to 4 antibiotics, on the other hand having no band showed resistant to 3 antibiotics thus the plasmid profiles observed in this study indicated that the plasmids are distributed at random in these strains and there was no notable correlation between antibiotic resistance and plasmid presence. In most of the cases, strains having similar antibiotic sensitivity patterns had different plasmid profile, implying that plasmid may not have any role in occurrence of resistance in the isolate investigated. During the present study, all the *Citrobacter* isolates showed rising trend of resistance against different antibiotics commonly prescribed by physicians for the treatment of *Citrobacter* infections. The difference in resistance profiles among *Citrobacter* isolates in the ecological niche clearly reflects the selective pressure of MAR index and plasmid profiling. The bacterial isolates viz. BGCC#2064, BGCC#2066 and BGCC#2091 harbored a plasmid of 23,130 bp with MAR index of 0.722. It was remarkably noted that isolates were similar by means of plasmid profile and antibiogram analysis with similar MAR index i.e. 0.77 indicating that isolates are of human and fecal origin. This may be due to the fact that fecal discharges of human and animals carrying antibiotics in the riverine environment directly or indirectly cause continued selection pressure of resistant organism in water. On the other hand, BGCC#2095 and BGCC#2137 harbored plasmid of 2027 and 4361 bp respectively, indicated that the bacterial isolates were more discriminatory on the basis of plasmid profiling with MAR index of 0.722 and 0.833. The results demonstrated that these *Citrobacter* strains can persist for very long time due to multidrug resistance. They can disperse virulent genes thus constitutes an environmental reservoir harboring virulent multidrug resistant strains with high MAR index.

Conclusion

Results stress the role of environmental *Citrobacter* isolates in combination with unintentional antibiotic release, as potential recruitment pools for human pathogens. Moreover, most of the studies have been carried out almost exclusively with clinical strains; while there are only few reports on the pathogenic aspects of environmental strains carrying antibiotic resistance therefore environmental isolate harboring virulent genes were investigated for their antibiotic profile. Since the aquatic environment is implicated as the reservoir for these microorganisms and consequently may be responsible for their transmission in humans, it is obvious that detailed studies on the pathogenic potential of the environmental *Citrobacter* strains will certainly contribute to the understanding of virulence properties of these bacteria. This study indicates the changing susceptibility and the status of drug resistance profile and virulence markers of *Citrobacter* spp. found in fresh water of river Narmada in order to establish the importance of these significant pathogens in aquatic environment.

Acknowledgement

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Table 1: Antibiogram analysis of virulent *Citrobacter* isolates isolated from river Narmada

Bacterial Culture	Species	Antibiotic resistance profile	MAR index
BGCC#2059	*Citrobacter sedlakii*	CB, AO, CU,AC, CN, PT,AS, P, CZ, S, Co, Tb	0.666
BGCC#2061	*Citrobacter freundii*	CB, AO, CU, AC, PB, P, Ct, CZ	0.444
BGCC#2062	*Citrobacter freundii*	CB, AO, CU, AC, CN, PT, PB, AS, P, Tb, CZ	0.611
BGCC#2064	*Citrobacter koseri*	CB,AO,CU,AC,CN,PT,PB,G, AS, P, Ct, CZ, Tb	0.722
BGCC#2065	*Citrobacter braakii*	CB,AO,CU,AC,CN, PB, P,CZ, Tb	0.5
BGCC#2066	*Citrobacter freundii*	CB,AO,CU,AC,CN,PT,PB,G, AS, P, Ct, CZ, Tb	0.722
BGCC#2069	*Citrobacter koseri*	CB,AO,CU,AC,CN,PT,PB,AS, P, CZ, Tb	0.611
BGCC#2072	*Citrobacter freundii*	CB, AO, CU, AC, CN, PB, Co, AS, P, Ct, Tb	0.611
BGCC#2073	*Citrobacter freundii*	CB, AO, CU, AC, CN, PB, Co, AS, P, Ct, Tb	0.611
BGCC#2074	*Citrobacter freundii*	AO,CU,AC,CN,PB,G, P, Tb	0.444
BGCC#2079	*Citrobacter freundii*	AO,CU,AC,CN,PT, PB, G,S, P, Ct, CZ, Tb	0.666
BGCC#2080	*Citrobacter freundii*	AO,CU,AC,CN,PB, G, AS,P, Ct	0.5
BGCC#2083	*Citrobacter freundii*	AO,CU,AC,CN,PB, AS, P, Ct, CZ	0.5
BGCC#2090	*Citrobacter freundii*	AO, CN, PT, PB, P, Ct, CZ, Tb	0.444
BGCC# 2091	*Citrobacter freundii*	CB, AO, CU, AC, CN,PT, PB, Co, G, P, S, Ct, CZ, Tb	0.777
BGCC#2092	*Citrobacter freundii*	CB, AO, AC, PT, PB, Co, G, S, P, Ct, Tb	0.611
BGCC#2093	*Citrobacter freundii*	AO, CU, AC, CN,PB, Co, G, AS, P, Ct, CZ, Tb	0.666
BGCC#2095	*Citrobacter freundii*	CB, AO, CU, Cf, CN, PT, PB, Co, S, P, Ct, CZ, Tb	0.722
BGCC#2101	*Citrobacter freundii*	AO,PB, Co, G, S, P, Ct, CZ, Tb	0.5
BGCC#2107	*Citrobacter koseri*	AO, CN,PB, P, Ct, CZ, Tb	0.388
BGCC#2108	*Citrobacter freundii*	AO, CN, Cf, PB, G, P, Ct, CZ, Tb	0.5
BGCC#2109	*Citrobacter koseri*	AO,PB,G, S, P, Ct, CZ, Tb	0.444
BGCC#2111	*Citrobacter freundii*	AO, PB, P, CZ, Tb	0.277
BGCC#2115	*Citrobacter freundii*	AO, CN,PB, P, Ct, CZ, Tb	0.388
BGCC#2118	*Citrobacter koseri*	AO, P, Ct, CZ, Tb	0.277
BGCC#2121	*Citrobacter koseri*	AO,PB, P	0.166
BGCC#2125	*Citrobacter braakii*	AO, CU, AC, CN, PT, PB, G, S, P, CZ, Tb	0.611
BGCC#2130	*Citrobacter freundii*	CB, AO, CU, AC, CN,PB, Co, AS, P, Ct, CZ, Tb	0.666
BGCC#2132	*Citrobacter freundii*	Tb, Ct, CZ.	0.166
BGCC#2133	*Citrobacter freundii*	CB, AO, CU, AC, CN,PB, Co, Ct, CZ, Tb	0.555
BGCC#2134	*Citrobacter freundii*	CB, AO, CU, AC, CN, PT, Co, AS, P	0.5
BGCC#2135	*Citrobacter freundii*	CB, AO, CU, AC, CN, PB, Co, G, AS, S, P.	0.611
BGCC#2136	*Citrobacter freundii*	CB, AO, AC, PT, Co, P	0.166
BGCC#2137	*Citrobacter freundii*	CB, AO, CU, AC, CN,PT, PB, Co, G, AS,S, P, Ct, CZ, Tb	0.833
BGCC#2138	*Citrobacter freundii*	AO, AC, CN, PB, Co, G, S, P	0.444
BGCC#2139	*Citrobacter freundii*	CB, AO, AC, PT, Co, P	0.333
BGCC#2140	*Citrobacter freundii*	CB, AO, AC, PT, Co, P	0.166
BGCC#2141	*Citrobacter freundii*	AO, CN, PB, G,AS,S,P	0.388
BGCC#2143	*Citrobacter freundii*	AO, CN,PB,G,S,P	0.333
BGCC#2146	*Citrobacter koseri*	CB,AO,CU,AC,CN,PB, Co, AS, P, Ct, CZ, Tb.	0.666
BGCC#2147	*Citrobacter freundii*	CB, AO, CU,AC, CN, PT, PB, AS, P, Ct, CZ, Tb, Ak	0.722
BGCC#2150	*Citrobacter freundii*	CB, AO, CU,AC, CN, PT, PB,AS, P, Ct, CZ, Tb	0.666
BGCC#2152	*Citrobacter braakii*	CB, AO, CU,AC, CN, PT, PB, AS, P, Ct, CZ, Tb	0.666
BGCC#2156	*Citrobacter freundii*	CB, AO, CU,AC, CN, PT, PB, AS, P, Ct, CZ, Tb	0.666
BGCC#2157	*Citrobacter freundii*	CB, AO, CU,AC, CN, PT, PB, AS, P, Ct, CZ, Tb	0.666
BGCC#2161	*Citrobacter freundii*	CB, AO, CU,AC, CN, PT, PB, AS, P, Ct, CZ, Tb	0.666
BGCC#2165	*Citrobacter freundii*	CB, AO, CU,AC, CN, PT, PB, AS, P, Ct, CZ, Tb	0.666
BGCC#2167	*Citrobacter freundii*	CB, AO, CU,AC, CN, PT, PB, AS, P, Ct, CZ, Tb	0.666
BGCC#2169	*Citrobacter freundii*	CB, AO, CU,AC, CN, PT, PB, AS, P, Ct, CZ, Tb	0.666
BGCC#2172	*Citrobacter braakii*	CB, AO, CU,AC, CN, PT, PB, AS, P, Ct, CZ, Tb	0.666
BGCC#2173	*Citrobacter werkmanii*	CB, CU,AC, CN, PT, PB, P, CZ	0.444

CB: Carbenicillin, AO: Aztreonam, CU: Cefuroxime, AC: Amoxyclave, CN: Cephoxitin, PB: Polymixin B, AS: Ampicillin/Sulbactam, S: Streptomycin, Ct: Ceftazidime, CZ: Cefazolin Tb: Tobramycin, P: Penicillin G, CO: Cotrimoxazole, G: Gentamicin, Cf: Ciprofloxacin, PT: Piperacillin/Tazobactam, MR: Meropenem, Ak: Amikacin. BGCC: Bacterial Germplasm Collection Center

Table 2: Plasmid profile of *Citrobacter* spp. isolated from fresh water of river Narmada

Bacterial Culture	No. of Bands	Size (in bp)
BGCC#2172	3	2322, 4361, 9416
BGCC#2061	3	4361, 6557, 9416
BGCC# 2139, 2079, 2135, 2137	1	4361
BGCC#2125	2	2322, 4361
BGCC#2111	5	~4000, 4361, 6557, 9416, 23130
BGCC#2083	2	~5500, 6557
BGCC#2141	5	4361, ~5000, 6557, 9416, 23130
BGCC#2165	5	4361, 6557, 9416, 23130, <23130
BGCC#2173	2	~3000, 9416
BGCC#2140	4	2322, 4361, 23130, <23130
BGCC#2134	2	4361, 9416
BGCC#2090, 2095	1	2027
BGCC# 2108	1	~2000
BGCC#2109	2	~5000, 23130
BGCC#2115	3	6557, 9416, 23130
BGCC#2118	2	9416, 23130
BGCC#2107	3	9416, 23130, <23130
BGCC# 2064, 2066, 2062, 2069, 2152, 2065, 2156, 2161, 2157, 2121, 2143, 2097, 2092, 2093, 2101, 2091	1	23,130

BGCC: Bacterial Germplasm Collection Center

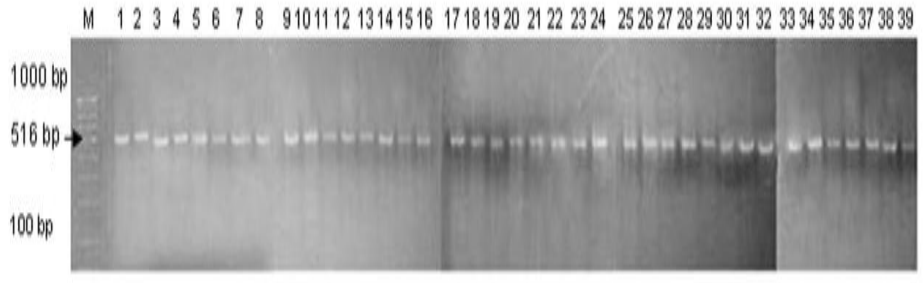

Fig. 1. Uniplex PCR analysis of *via* B gene among *Citrobacter freundii* isolates isolated from river Narmada.

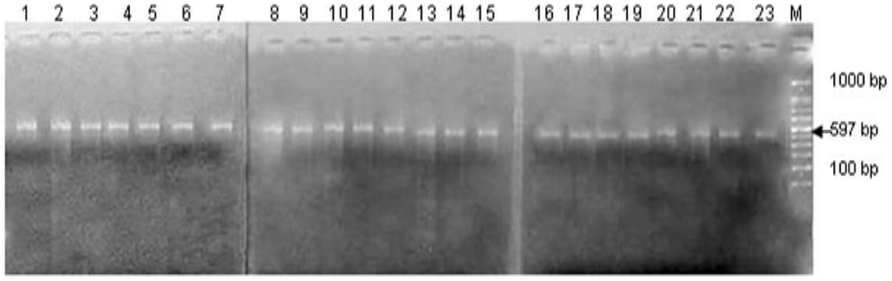

Fig. 2. Uniplex PCR analysis for *hly* A gene of *Citrobacter* isolates isolated from river Narmada.

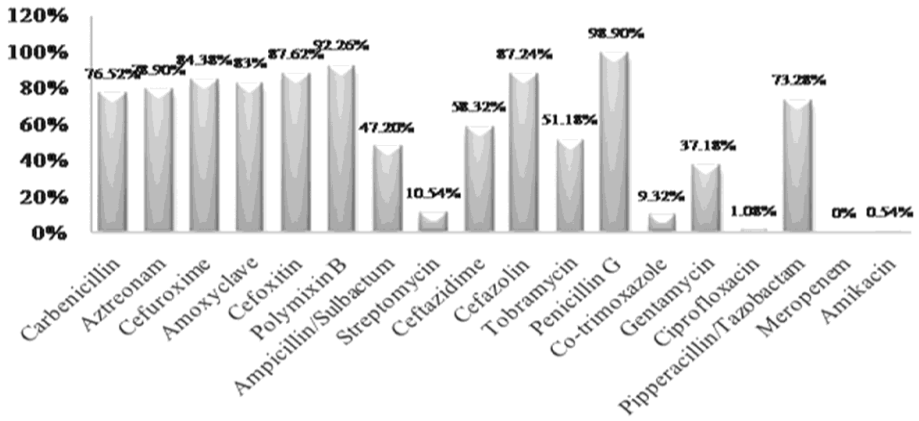

Fig. 3. Antibiotic resistance percentage of virulent *Citrobacter* isolates isolated from river Narmada.

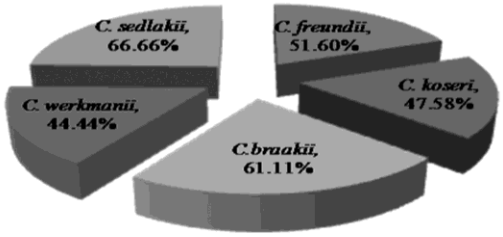

Fig. 4. Multi drug resistance pattern exhibited by different virulent *Citrobacter* genomospecies isolated from river Narmada.

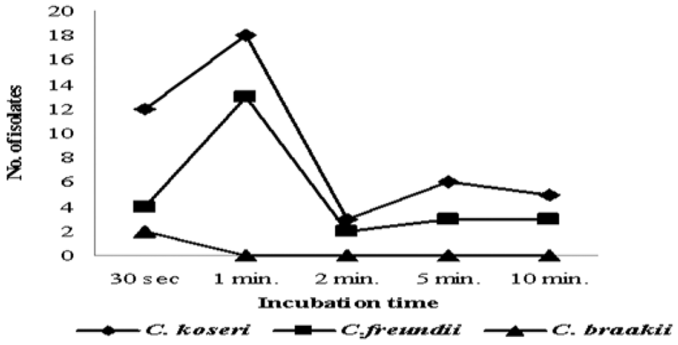

Fig. 5. β-lactamase activity among the *Citrobacter* isolates isolated from river Narmada.

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