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Real-Time PCR based Rapid and Culture-Independent Detection of *Citrobacter:* 'An Alarming State for Environment and Human Health'.

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ABSTRACT

The changing global scenario of environmental conditions and altered microbial community has severely deteriorated the quality of water which enforced an elevated health risks on human beings due to unavailability of safe and pristine water. *Citrobacter* is one of the fecal coliform bacteria of family *Enterobacteriaceae.* It is an opportunistic pathogen formerly used as an indicator bacterium along with other fecal coliforms namely *Escherichia coli, Klebsiella* and *Enterobacte*r, but is now known to cause several diseases in human beings. In present study we collected 135 samples from potable water and riverine-systems and confirmed the existence of *Citrobacter* using the conserved sequence of *gnd* (6-phosphogluconate dehydrogenate) gene with the application of SYBR green real-time PCR, which demonstrated extremely high specificity, fidelity and stringency in detection strategy. The *Citrobacter* counts in potable water were in the range of zero to 1.54 x10^4 ± 572.88 cfu/100ml. The *Citrobacter* levels in surface water were 2.18 x10^4 ±848, 1.54 x10^4 ±462, 3.53 x10^4 ±1299, 2.57 x10^4 ±912, 8.78 x10^3 ±201, 1.28 x10^4 ±370, CFU/100ml; associated with hydrophytes were 6.24 x10^4 ±1535, 8.42 x10^4 ±2711, 1.19 x10^5 ±4792, 1.12 x10^5 ±4177, 8.32 x10^4 ±2329, 8.08 x10^4 ±2149 CFU/10g and in the sediments the counts were 2.50 x10^4 ±647, 5.02 x10^4 ±1852, 3.26 x10^4 ±1186, 7.65 x10^4 ±2884, 1.50 x10^4 ±355, and 2.56 x10^4 ±737 CFU/10g at sampling site # 1, site # 2, site # 3, site # 4, site # 5 and site # 6, respectively. The present assay could be applied in the detection and regular monitoring of potable water and other environmental samples to check waterborne diseases and outbreaks caused by *Citrobacter*.

KEYWORDS: fecal coliforms, microbial contamination, real-time PCR, *Citrobacter*, pathogenic bacteria

1. INTRODUCTION

The world's environmental conditions are changing with faster rate and leading to a variety of problems imposed on living as well as nonliving components of ecosystem. The aquatic ecosystems have been more severely affected than that of others. The polluted water reservoirs have led to the inappropriate distribution of microflora and increased pathogenic bacterial population. Hence, the availability of fresh potable water has become a global challenge. Therefore, it becomes utmost essential to check the bacteria present in potable water. It is impractical to check all the pathogenic bacteria, hence, some indicator microorganisms are required to assess the microbially safe quality of water. Fecal coliforms bacteria namely *Escherichia coli, Enterobacter, Klebsiella* and *Citrobacter,* are most commonly used as the indicator bacteria.

Citrobacter, a genus of family *Enterobacteriaceae*, is currently divided into 11 species, [1] once thought to be an indicator bacterium but now known to cause several diseases to human beings. It is also reported that *Citrobacter* species are found in the nasal passages, cerebrospinal fluid, brain, urinary tract, and is believed to be a commensal in the human and animal intestines. *Citrobacter freundii* and other *Citrobacter* species known to cause urinary tract infection (UTI), wound infection, septicemia, meningitis, and endocarditis in adults, as well as septicemia, meningitis, brain abscesses in neonates [2,3].

The present studies have been conducted in Lucknow city (latitude, 26.28 N; longitude, 80.24 E; altitude, 126 m and have population more than 5 million according to census-2011) the state capital of Uttar Pradesh. River Gomti, a major tributary of river Ganga, is the life-

line of Lucknow as it meets about half of the total domestic water demand (about 550 mLd) of the town [4]. However, river Gomti is recognized as one of the severely polluted rivers in India and contain enormous amount of organic and inorganic wastes from point and non-point sources leading to eutrophication of the river water [4] and hence flourish bacteria.

Analysis for fecal-indicator bacteria by MPN (most probable number) procedures provides an indication of fecal pollution in water [5]. However, 48 h are required to confirm fecal pollution in a sample and provides no insight about the types of coliform bacteria [6]. Hence, in this study we have targeted for the first time the *gnd* gene encoding enzyme of the pentose-phosphate pathway (6-phosphogluconate dehydrogenase: 6-PGDH, EC 1.1.1.44) [7] for the enumeration and detection of *Citrobacter* in potable water and riverine systems. The fast and reliable real-time PCR based on SYBR Green probe [8] have employed in this study. The present study could be used in the assessment and regular monitoring of water reservoirs to check outbreaks caused by *Citrobacter*.

2. MATERIALS AND METHODS

2.1 Designing of primers specific to *gnd* gene

The complete coding sequences of *gnd* gene (Accession Numbers: U14432, U14424, U14425, U14427, U14429) were retrieved from NCBI GenBank database (http://www.ncbi.nlm.nih.gov) to design a set of primers for specific detection of *Citrobacter* in environmental samples.

To determine the conserved sequences for designing the primers against *gnd* gene, an alignment of the sequences were done using ClustalW program (www.ebi.ac.uk/clustalW). A set of primers for *gnd* gene were designed in highly conserved region of the sequence through Beacon Designer 5.0, Premier Biosoft International (Table 1). The specificity of primers were analyzed against known microbial genomes and sequences by BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/BLAST/) to make it sure that no homology was observed with known gene sequences of other water-borne pathogens. The synthesis of all the primers for this study was carried out by Metabion (Gmbh, Germany).

Table 1. Primers used in detection of *Citrobacter* species in riverine samples and potable water

Gene	Primers Sequences (5'-3') **F**: Forward; **R**: Reverse	Length (bp)	Tm (°C)	GC (%)	Product size (bp)
gnd	**F**: GCGTTCTATTGTCAGCGTTG	20	55.0	50.0	98
	R: TTAATCATAGACTGCGAGTGTTG	23	54.4	39.1	

2.2. Generation of standard curve for quantitative enumeration of *Citrobacter* species

To prepare the standard curve (Figure 3), the reference strain (*Citrobacter* MTCC 1657 carrying *gnd* gene acquired from Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology (IMTECH), Chandigarh, India) was cultured in triplicate in 15 ml Luria–Bertani (LB) broth for 12 h at 37± 1°C to approximately optical density of 0.8 at 600 nm. The number of colony forming units (CFU/ml) of cell suspension was determined by plating 100 µl of the three-fold diluted culture onto five Luria–Bertani agar plates. The average number of colony forming units from the five plates was used to calculate the concentration (CFU/ml) of cells in the culture. Further, the triplicate cultures of reference strain (optical density 0.8 at 600 nm) were serially diluted ten-fold to yield 10^7 down to 1 CFU/ml in phosphate–buffered saline as estimated by standard plate count method. DNA template was prepared from each dilution as per Jyoti *et.al*. (2010) [9] and real-time PCR assays for *gnd* gene were performed using an iCycler (BIO-RAD, USA) real-time PCR instrument and Quantifast SYBR Green PCR kit (Qiagen, Germany). Briefly, the reaction mixture contained 0.2 µM of the forward and 0.2 µM of the reverse oligo-nucleotide primers (1 µl each) for *gnd* gene, 12.5 µl of Quantifast SYBR Green PCR kit (Qiagen, Germany), deionized water (5.5 µl nuclease free water provided by Qiagen, Germany) and 5 µl DNA template (corresponding to 10^6–1 CFU/PCR) in a final volume of 25 µl. For the negative controls 5 µl sterilized Milli-Q water used as template. The PCR amplification protocol consisted of an initial denaturation of 5 min at 95 °C, followed by 45 cycles of three steps consisting of 10 s at 95 °C, 20 s at 55 °C and 20 s at 72 °C. The fluorescence signals were measured at the end of each extension step. Melting temperature (Tm) analysis of the products was performed by reducing the temperature to 65 °C and then heating to 95 °C at a rate of 0.2 °C /second after the completion of PCR amplification. Fluorescence signals were on continuous surveillance to confirm amplification specificity. The Tm peaks were calculated based on the initial fluorescence curve (F/T) by plotting the negative derivative of fluorescence over temperature versus temperature (-dF/dT versus T). To generate standard curves for *gnd* gene the real-time PCR protocol were repeated thrice and average PCR efficiencies and R^2 values were calculated to check the reproducibility of the assays.

2.3 Quantitative enumeration of *Citrobacter* in potable water

For potable water supply to Lucknow city, water of river Gomti is pumped from Gaughat, and is sent through a

pipeline to Lucknow Jal Sansthan Aishbagh, 4 km away, where water purification is carried out by alum treatment, filtration, and chlorination prior to being released into the drinking water supply [10, 11, 12]. To investigate the risk of contamination of potable waters by *Citrobacter* due to defective water distribution systems and insufficient treatment during production, 1 liter of water samples were collected in triplicate for culture free quantitative enumeration at ten sites. Site: A, Gaughat (raw water intake point of river Gomti) site : B, Aishbagh waterworks (before water enters to distribution system); site : C , Hussainganj; site : D, Kaiserbagh (water-distribution pipeline that neither percolated nor ran along open drainage); site : E, Nakkhas; site : F, Shashtri Nagar, site : G, Saadatganj; site : H, City Station; site : I, Moti Nagar (pipeline that percolated and ran along open drainage); site : J, KGMU (Figure 1). The water samples were collected into sterile glass bottles, stored on ice and transported to the laboratory for analyses within 6 h on the same day [13] in densely populated areas across the urban boundaries of Lucknow city. The numbering of sites was in order of the sample collection. Bacteriological qualities of potable water samples, collected in this study were analyzed to determine *Citrobacter* contamination. Multigenomic DNA from water (500 mL) was extracted by boiling preparation method as per procedure described by Jyoti *et al.* (2010) [9]. The quantification of *Citrobacter* based on *gnd* gene were carried out by ten-fold standard curve generated by diluting the culture of *Citrobacter* MTCC 1657.

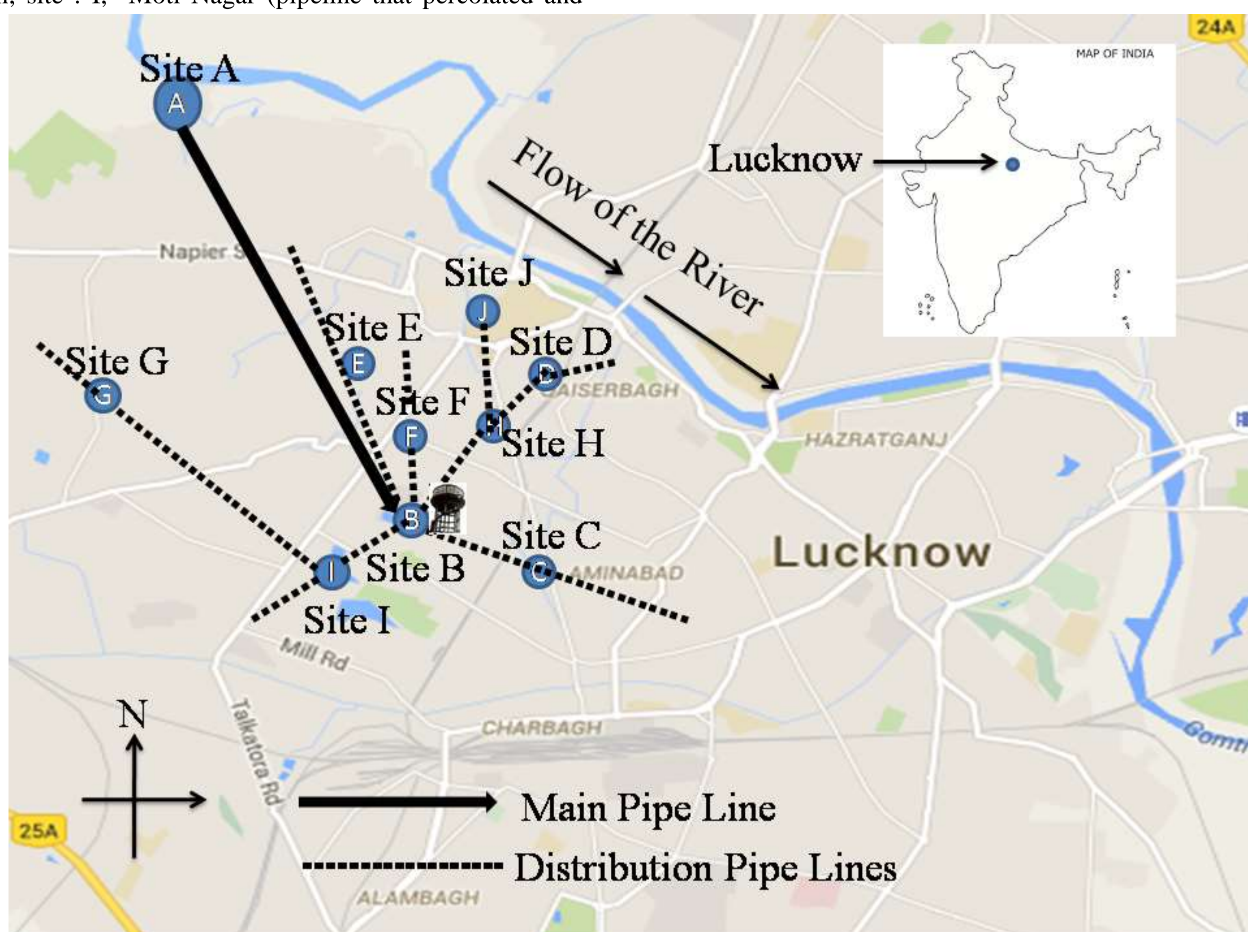

Figure 1. Sampling sites of potable water in Lucknow city.

2.4 Quantitative enumeration of *Citrobacter* in Riverine Systems

2.4.1 Sampling of Surface Water from Riverine Systems and DNA Isolation

The study was conducted around 20 km stretch of river Gomti in Lucknow city (Figure 2). River Gomti confluence with river Ganga after traversing a distance of around 730 km in the Indo-Gangetic alluvial region. Although, this river contains water throughout the year but except the monsoon season it exhibits sluggish flow. In this study, six sampling sites were selected in up-to-downstream along the river Gomti based on various human activities on the banks such as Ghaila-Bridge (site# 1, located in upper-most stream of river from where river enters the Lucknow city), Gaughat (site# 2, washing and bathing spot), Shaheed Smarak (site# 3, bathing and recreation spot), Bhaisakund (site# 4, human cremation spot), Chandiamau (site# 5, bathing and human activities) and Indira jal Setu (site# 6, down-most stream location). A cross-sectional approach was used to collect river water samples. Samples were collected

in triplicate at each site (n=9) from six locations situated in up-to-down-stream fashion. In brief, three transects were established randomly at each site and one liter water samples were collected from 30 cm below the water surface from left bank, mid-stream and right bank of the river. Water samples were stored in sterile glass bottles, labeled and transported on ice to the laboratory for further analysis. The processing and analysis were carried out within 6 h after sample collection. DNA isolation was carried out by boiling preparation method.

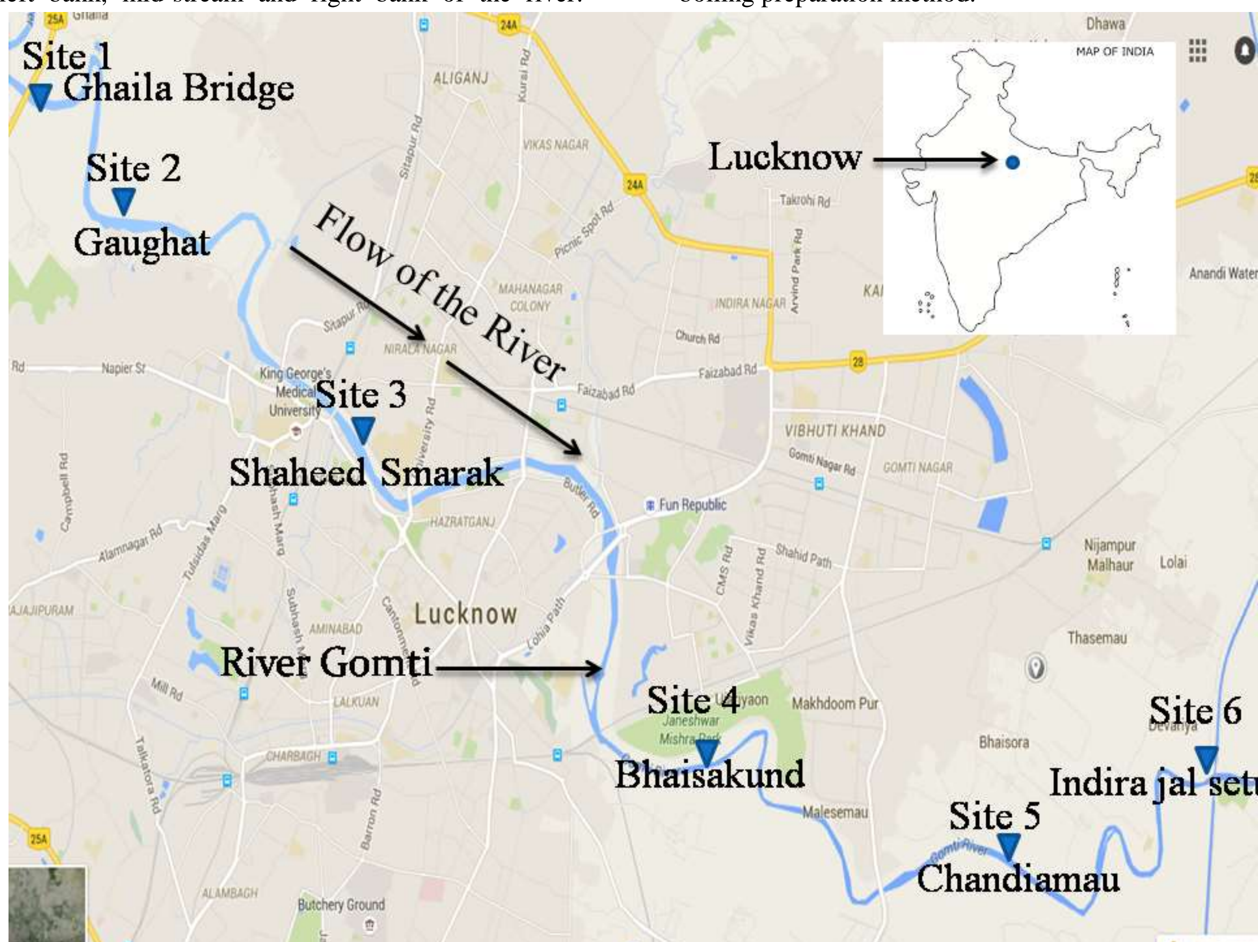

Figure 2 Map of sampling sites of surface water, plants and sediment in river Gomti in Lucknow city. The figure was taken and redrawn from Map of India-Google map (map not to scale).

2.4.2. Collection of Hydrophytes from Riverine System and DNA Isolation

Same sampling sites (Figure 2) were selected and three transects were prepared randomly to collect hydrophytes. The submerged hydrophytes *Potamogeton crispus* (L.) were collected as per APHA protocol [13]. Along each transect, 1 m^2 quadrats were established at a distance of 0.5 m from left and right banks. All the *P. crispus* (n=6) within the quadrats at each sampling sites were collected and pooled in sterile zip-lock bags. Further, within 6 h the hydrophytes associated microbiota were recovered in 500 mL phosphate buffered saline. A 10 and 100-fold diluted portion of this sample was used for estimation of *Citrobacter* in CFU/10g of hydrophytes. In brief, *P. crispus* (10 ± 0.5 g fresh weight) were sonicated for 30 sec in 350 ml phosphate buffered saline and then kept on rotatory shaker (INNOVA 4230) for 10 minutes to release the plant associated microbiota. Subsequently plants were rinsed thrice in 50 ml phosphate buffered saline. The sonication (amplitude: 30%, pulse time: 0.5 sec.; UP200S Ultrasonic processor, dr. hielscher GmbH, Germany) of hydrophytes followed by three subsequent rinsing recovered an average of 95% of bacteria from plant surface. Further, the rinset which mostly contain bacteria was then carefully removed for isolation and purification of multigenomic DNA using boiling lysis followed by precipitation by sodium acetate-ethanol method. The quantity and quality of isolated DNA was measured by NanoDrop spectrophotometer ND 3.0 1000 (NanoDrop Technologies, Wilmington, DE, USA).

2.4.3. Collection of Sediments from Riverine System and Isolation of Multigenomic DNA

Same sampling sites as for surface water and hydrophytes were selected for collection of sediments in river Gomti (Figure 2). The sediment core samples (~250 g) were collected directly from an approximately 0.5 cm below the bottom of the river at three different points (~1m

distance) of each sampling site from both banks of river using a sterile stainless steel spatula and kept in sterile plastic bags. All the six samples (n=6) from each site were pooled and mixed to prepare the grab sample. Finally 10 ± 0.5 g fresh weights from each site were added to 100 ml of sterile phosphate-buffered saline and vortexes. Further, the vortexed samples were placed in incubator shaker (INNOVA 4230) at 220 rpm at 37 ^{0}C for 10 minutes and then allowed to settle by gravitational forces at the base of the tube. Now the supernatant which mostly contain bacteria were cautiously separated for isolation of multigenomic DNA using boiling lysis and precipitation by sodium acetate-ethanol procedure. The purity and content of extracted DNA was analyzed by NanoDrop spectrophotometer ND 3.0 1000 (NanoDrop Technologies, Wilmington, DE, USA).

2.5 Statistical Analyses

For the comparison of PCR amplification efficiencies and detection sensitivities among different experiments, slopes of the standard curves were calculated by performing a correlation and regression analysis through iCycler iQ^{Tm} Real-Time Detection System Software Version 3.0 A. Amplification efficiency (E) was estimated by using the slope of the standard curve and the formula $E = (10^{-1/slope})-1$. An experiment with theoretical 100% efficiency will generate a slope of -3.322. Data obtained from conventional and qPCR were compared by Wilcoxon Rank-SumTest. *Citrobacter* species retrieved in this study at different sites were analyzed using one way analysis of variance [14].

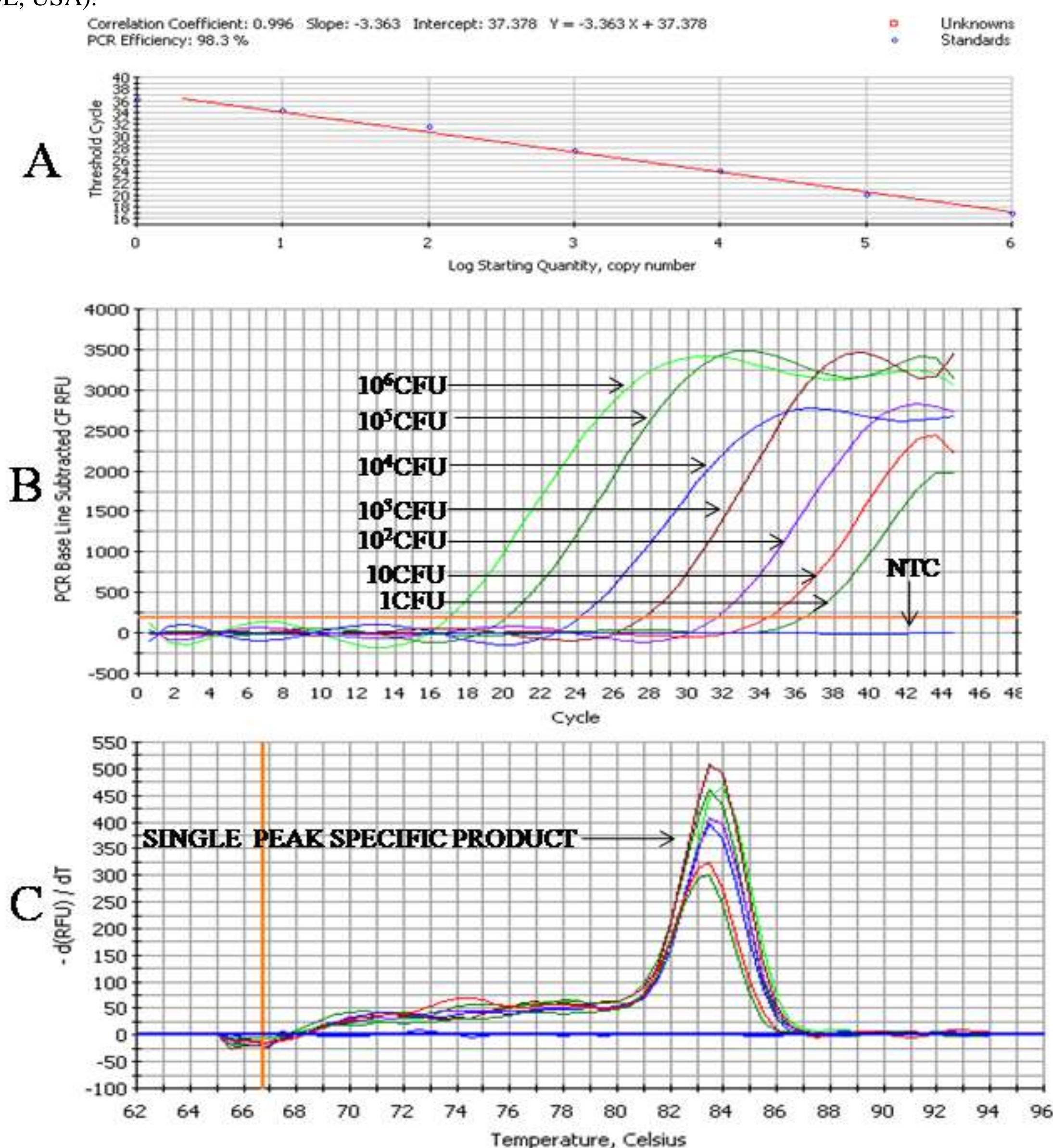

Figure 3 PCR profiling and melting curve analysis of qPCR (A-C). A: Standard curve, B: Amplification curve, C: Melt curve. Product was (product length 98 bp) amplified targeting *gnd* gene of 10-fold serially diluted culture of *Citrobacter koseri* MTCC 1657.

3. RESULTS

The real-time PCR strategy based on SYBR Green probe targeting *gnd* gene were applied to detect *Citrobacter* quantitatively in potable waters and different environmental samples collected from river Gomti in Lucknow. The result demonstrate that the contamination levels of *Citrobacter* at various sampling sites varied significantly (one way ANOVA, $p<0.05$).

Potable water samples collected from sites: E, G, I and J were located in water distribution system that either percolated or ran along open drainage, exhibited the presence of *Citrobacter* species (Table 2). The *Citreobacter* levels at sites: A-J targeting *gnd* gene were 1.54 $x10^4$ ± 572.88, ND (not detected), ND, ND, 2.30 $x10^3$ ±66.01, ND, 6.56 $x10^3$ ±211.32, ND, 3.10 $x10^2$ ± 9.02 and 6.08 $x10^3$ ± 189.70 CFU/100 ml, respectively.

Table 2. Culture-free detection and enumeration of *Citrobacter* species in potable water by qPCR

[a]Sampling Sites	Site Name	(*Citrobacter* /100mL)[bc]
Site : A	Gaughat	1.54 $x10^4$ ± 572.88
Site : B	Aishbagh	ND[d]
Site : C	Husain Ganj	ND
Site : D	Kaiserbagh	ND
Site : E	Nakkhas	2.30 $x10^3$ ±66.01
Site : F	Shastrinagar	ND
Site : G	Saadatganj	6.56 $x10^3$ ±211.32
Site : H	City Station	ND
Site : I	Motinagar	3.10 $x10^2$ ± 9.02
Site : J	KGMU	6.08 $x10^3$ ± 189.70

[a]site A: raw water intake point in the river Gomti, site B: Aishbagh Waterworks before water enters the distribution system; sites C, D, F and H: water-distribution pipeline that neither percolated nor ran along open drainage; sites E, G, I and J: pipeline that percolated and ran along open drainage, [b]nuclease-free water used as negative Control, [c]One way ANOVA, $p < 0.05$ for each column, [d]ND: not detected.

Surface water is the potent source of microorganisms since it carries dissolved as well as suspended organic and inorganic nutrients. The levels of *Citrobacter* contamination in surface water were 2.18 $x10^4$ ±848, 1.54 $x10^4$ ±462, 3.53 $x10^4$ ±1299, 2.57 $x10^4$ ±912, 8.78 $x10^3$ ±201, 1.28 $x10^4$ ±370, CFU/100ml at sampling site # 1, site # 2, site # 3, site # 4, site # 5 and site # 6, respectively (Table 3).

Hydrophytes play significant role to provide the stationary/static habitats of bacteria in river water. Bacteria get attached to plants parts for example leaves, stems or on the roots of the floating plants. The levels of *Citrobacter* contamination associated with hydrophytes were 6.24 $x10^4$ ±1535, 8.42 $x10^4$ ±2711, 1.19 $x10^5$ ±4792, 1.12 $x10^5$ ±4177, 8.32 $x10^4$ ±2329, 8.08 $x10^4$ ±2149 CFU/10g at sampling site # 1, site # 2, site # 3, site # 4, site # 5 and site # 6, respectively (Table 3).

Table 3. *Citrobacter* **species** counts in river Gomti in surface water (per 100 ml), hydrophytes (per 10 g) and sediments (per 10 g)

Sampling sites	Sites Name	Surface water	Hydrophytes	Sediments
Site # 1	GhailaBridge	2.18 $x10^4$ ±848	6.24 $x10^4$ ±1535	2.50 $x10^4$ ±647
Site # 2	Gaughat	1.54 $x10^4$ ±462	8.42 $x10^4$ ±2711	5.02 $x10^4$ ±1852
Site # 3	Shaheed Smarak	3.53 $x10^4$ ±1299	1.19 $x10^5$ ±4792	3.26 $x10^4$ ±1186
Site # 4	Bhaisakund	2.57 $x10^4$ ±912	1.12 $x10^5$ ±4177	7.65 $x10^4$ ±2884
Site # 5	Chandiamau	8.78 $x10^3$ ±201	8.32 $x10^4$ ±2329	1.50 $x10^4$ ±355
Site # 6	Indira Jal Setu	1.28 $x10^4$ ±370	8.08 $x10^4$ ±2149	2.56 $x10^4$ ±737

The sediments are the potent and silent reservoir of microorganisms in the rivers. By analyzing the sediments the inflow and discharge of drainage system into the river can be predicted. Sediments can also be one of the important factors which can be helpful in microbial source tracking. The *Citrobacter* were found in sediments in the range of 2.50 $x10^4$ ±647, 5.02 $x10^4$ ±1852, 3.26 $x10^4$ ±1186, 7.65 $x10^4$ ±2884, 1.50 $x10^4$ ±355, and 2.56 $x10^4$ ±737 CFU/10g at sampling site # 1, site # 2, site # 3, site # 4, site # 5 and site # 6, respectively (Table 3).

4. DISCUSSION

The bacteria of Genus *Citrobacter* are Gram-negative, non-spore forming, facultative anaerobic and motile bacilli having peritrichous flagella for locomotion [15]. Some member of this genus can be opportunistic pathogens to immunocompromised hosts. They typically utilize citrate as their sole carbon source. They do not have oxidase but have catalase and are methyl red positive. *C. rodentium* belongs to the family human and animal pathogens that use attaching and effacing (A/E) lesions to colonize the host gastrointestinal tract [16]. Attaching and effacing (A/E) types of lesions are characterized as the destruction of brush border microvilli and attachment of bacteria to the plasma membrane of the epithelial cells of the hosts. A/E lesions capacity is mainly encoded by a pathogenicity island which is termed as locus of enterocyte effacement (LEE) [17]. *Citrobacter* strains are widely distributed in environment and are found in soil, sewage, water, foodstuffs, various animals, birds and cattle.

The present study is based on q-PCR assay targeting *gnd* gene of *Citrobacter* species. The detection limit of this q-PCR assay was up to 1 CFU/PCR (Figure 3) with PCR efficiency 98.3 % which exhibit the high accuracy and fidelity of the technique and protocol. The advantage of low limit of detection (LOD) by q-PCR could be applied in providing safe and potable water as well as monitoring and management of outbreaks caused by *Citrobacter.*

Two realms of the environments were taken into consideration for this study. The first realm was potable water. The *Citrobacter* were observed at various sampling sites. Site: A represents surface water of river Gomti whereas, sites: B-J represent potable water. At sampling sites: B, C, D, F and H *Citrobacter* were not detected since at these sites the potable water pipe-line supply were neither percolated nor ran along open drainage system. At sampling sites: E, G, I and J the *Citrobacter* were detected because at these sites pipe-line supply was either percolated or ran along open drainage systems. Hence, the possessions of *Citrobacter* in potable waters at some sampling sites were due to local pollution.

The second realm targeted for this study was the riverine systems. The presence of *Citrobacter* in various environmental samples endorses its wide distribution in the nature. Earlier it was reported that the members of family *Enterobacteriaceae* namely *Escherichia, Enterobacter, Citrobacter,* and *Klebsiella* were the most commonly encountered genera in shell egg processing plants [18]. The effluents from various kinds of food processing industries carrying waste materials discharge into riverine systems. Furthermore, various point and non-point sources of pollution enhance the bacterial population in the river. Some of the microorganisms get settled down with sediments; some of them may adhere on hydrophytes, while remaining gets suspended in water bodies.

The *Citrobacter* populations were identified in all the three domains of the river (1) surface water, (2) hydrophytes and (3) sediments. Although, the natural water reservoirs have self-sustenance potential to maintain ecological balance through various mechanisms including solar inactivation of pathogens by UV-B solar radiation and interaction of sunlight generated reactive oxygen species (ROS) with external sensitizer molecules [19]. At various trophic levels of riverine ecosystem, the survival rates and die-offs of the biological contaminants are also governed through grazing and predation [20,21,22]. However, the unmanaged anthropogenic waste disposal into the small tributary rivers has overburdened these natural resources leading to water-borne diseases and outbreaks. The detection of *Citrobacter* species in river water, sediment and hydrophytes is important to study their function in spread and persistence of antimicrobial resistance [23]. Earlier research on persistence of fecal indicator bacteria *E. coli* and *Enterococci* in mats of the green alga *Cladophora glomerata* (L.) kütz have concluded that such associations serve as environmental source of indicator bacteria [24].

Earlier investigations revealed that the value of total coliform in an environmental sample consists of *E. coli, Citrobacter*/*Enterobacter* and *Klebsiella* were in ratio 94:4:2 [25, 26]. But in this study the fecal coliforms ratio in surface water (n=54) from six sampling sites were 88.89: 4.78: 5.11: 1.52 of *E. coli* : *Enterobacter* : *Citrobacter* : *Klebsiella* respectively. We observed that the contamination levels of *Citrobacter* in surface water of river Gomti were two to five higher orders of magnitude (Table 3) and the pattern of contamination were in increasing order from upstream to downstream fashion.

Aquatic flora could be an important and significant nonpoint source of pollution [27]. Earlier investigations reveal the occurrence and growth bacteria on hydrophytes [28,29,30,]. Further, bacteria adhered on hydrophytes could be released frequently into water-stream by wave action on the river banks or by anthropogenic activities and could float several miles downstream along the course of the river and get transported to distant destinations. The analysis of (n=36) hydrophytes depicts that the percent ratio of *E. coli* : *Enterobacter* : *Citrobacter* : *Klebsiella* were 83.44: 10.51: 3.64: 2.42 respectively.

Sediments play an important role in determining the types of contamination in the rivers since they are the silent reservoir of the microbial community. The large numbers of microorganisms are attached to suspended particles and sediments [31,32]. Since the heavy particles that cannot flow in water get settled down at the bottom of the river. Hence, the microflora along with these particles also get settled down. In this study we observed that the

number of *Citrobacter* counts were four to five higher orders of magnitude (Table 3). By analyzing the sediment samples (n=36) from six sampling sites the relative percent ratio of *E. coli* : *Enterobacter* : *Citrobacter* : *Klebsiella* were 60.22 : 7.39: 26.69 : 5.74, respectively. This result delineate that *Citrobacter* counts in sediments were much more predominant after *E.coli*.

From this study we can conclude that the levels of all the fecal coliforms were minimum at sampling site # 1 (upstream) whereas the community were maximum at sampling site # 6 (downstream) and this progression were in increasing order with little exceptions. The bottom of river Gomti is mostly marshy due to its sluggish flow which leads to the depletion of oxygen and hence high biochemical oxygen demand (BOD) [4]. High BOD has led to change in the ecosystem of the river hence, the unusual distribution of microbial community was observed.

AUTHOR CONTRIBUTIONS

CP: Sample collected, executed the experiments, and designed the manuscript.

RU, RS: Designed and analyzed the experiment; SKP: Interpreted Data.

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REFERENCES

1. Brenner, D. J., O'Hara, C. M., Grimont, P. A., Janda, J. M., Falsen, E., Aldova, E., Ageron, E., Schindler, J., Abbott, S. L., and Steigerwalt. A. G. 1999. Biochemical identification of Citrobacter species defined by DNA hybridization and description of Citrobacter gillenii sp. nov. (formerly Citrobacter genomospecies 10) and Citrobacter murliniae sp. nov. (formerly Citrobacter genomospecies 11), J Clin Microbiol, 37, 2619-2624.
2. Brenner, D. J., Grimont, P. A., Steigerwalt, A. G., Fanning, G. R., Ageron, E., and Riddle, C. F. 1993. Classification of citrobacteria by DNA hybridization: designation of Citrobacter farmeri sp. nov., Citrobacter youngae sp. nov., Citrobacter braakii sp. nov., Citrobacter werkmanii sp. nov., Citrobacter sedlakii sp. nov., and three unnamed Citrobacter genomospecies, Int J Syst Bacteriol, 43, 645-658.
3. Samonis, G., Karageorgopoulos, D. E., Kofteridis, D. P., Matthaiou, D. K., Sidiropoulou, V., Maraki, S. and Falagas. M. E. 2009. Citrobacter infections in a general hospital: characteristics and outcomes, Eur J Clin Microbiol Infect Dis, 28, 61–68.
4. Singh, K. P., Malik, A., Mohan, D. and Sinha, S., 2004. Multivariate statistical techniques for the evaluation of spatial and temporal variations in water quality of Gomti River (India)-a case study, Water Res, 38, 3980-3992.
5. World Health Organization. 2002. The world health report 2002: Reducing risks, promoting healthy life, Geneva, Switzerland.
6. Ram, S., Vajpayee, P., Dwivedi, P. D., Shanker, R. 2011. Culture-free detection and enumeration of STEC in water, Ecotoxicol Environ Saf, 74, 551-557.
7. Nelson, K. and Selander, R. K., Intergeneric transfer and recombination of the 6-phosphogluconate dehydrogenase gene (gnd) in enteric bacteria, Proc Natl Acad Sci USA, 91, 10227-31, 1994.
8. Patel, C. B., Shanker, R., Gupta, V. K., and Upadhyay, R. S., 2016. Q-PCR, based culture-independent enumeration and detection of Enterobacter: an emerging environmental human pathogen in riverine systems and potable water. Front. Microbiol. 7:172.doi: 10.3389/fmicb.2016.00172.
9. Jyoti, A., Ram, S., Vajpayee, P., Singh, G., Dwivedi, P. D., Jain, S. K. and Shanker, R., 2010. Contamination of surface and potable water in South Asia by Salmonellae: Culture-independent quantification with molecular beacon real-time PCR, Sci Total Environ, 408, 1256–1263.
10. Ram, S., Singh, R. L. and Shanker, R., 2008. In-silico comparison of real-time PCR probes for detection of pathogens, In Silico Biol, 8, 251-259.
11. Clever, M., Jordt, F., Knauf, R., Räbiger N., Rüdebusch, M., and Hilker-Scheibel, R., 2000. Process water production from river water by ultrafiltration and reverse osmosis, Desalination, 131, 325–336.
12. Shrivastava, R., Upreti, R. K., Jain, S. R., Prasad, K. N., Seth, P. K. and Chaturvedi, U. C., 2004. Suboptimal chlorine treatment of drinking water leads to selection of multidrug-resistant Pseudomonas aeruginosa, Ecotoxicol Environ Saf, 58, 277–283.
13. APHA (American Public Health Association), Standard Methods for the examination of water and wastewater, 18th edition, American Public Health Association, Washington, D.C. 1998.

14. Gomez, K. A. and Gomez, A. A. 1984. Statistical Procedures for Agricultural Research, New York: Wiley.
15. Doran, T. I. 1999. The role of Citrobacter in clinical disease of children: review, Clin Infect Dis, 28, 384-394.
16. Mundy, R., Pickard, D., Wilson, R. K., Simmons, C. P., Dougan, G. and Frankel, G. 2003. Identification of a novel type IV pilus gene cluster required for gastrointestinal colonization of Citrobacter rodentium, Mol Microbiol, 48, 795-809.
17. McDaniel, T. K., Jarvis, K. G., Donnenberg, M. S. and Kaper, J. B. 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens, Proc Natl Acad Sci USA, 92, 1664-1668.
18. Musgrove, M. T., Northcutt, J. K., Jones, D. R., Cox. N. A., and Harrison, M. A. 2008. Eaterobacteriaceae and related organisms recovered from shell eggs in conimc'rcial facilities. Poult. Sci. 87:1211-1218.
19. Kohn, T. and Nelson, K. L., 2007. Sunlight-mediated inactivation of MS2 coliphage via exogenous singlet oxygen produced by sensitizers in natural waters, Environ Sci Technol, 41, 192-197.
20. Sinton, L., Hall, C. and Braithwaite, R., 2007. Sunlight inactivation of Campylobacter jejuni and Salmonella enterica, compared with Escherichia coli, in seawater and river water, J Water Health, 5, 357-365.
21. Sherr, E. B., and Sherr, B. F., 2002. Significance of predation by protists in aquatic microbial food webs, Antonie van Leeuwenhoek, 81, 293-308.
22. Peters, F., 1994. Prediction of planktonic protistan grazing rates, Limnol Oceanogr, 39, 195-206.
23. Sletvold, H., Johnsen, P. J., Simonsen, G. S., Aasnaes, B., Sundsfjord, A. and Nielsen, K. M., 2007. Comparative DNA analysis of two vanA plasmids from Enterococcus faecium strains isolated from poultry and a poultry farmer in Norway, Antimicrob Agents Chemother, 51, 736-739.
24. Whitman, R. L., Shively, D. A., Pawlik, H., Nevers, M. B., and Byappanahalli, M. N., 2003. Occurrence of Escherichia coli and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan, Appl Environ Microbiol, 69, 4714-4719.
25. Dufour, A. P., 1977. Escherichia coli: the fecal coliform. In: Hoadley, A. W., Dutka, N.J. (Eds.), Bacterial Indicators/Health Hazards Associated with Water, Am Soc Test Mater, Philadelphia, 48–58.
26. Leclerc, H., Mossel, D. A., Edberg, S. C. and Struijk, C. B., 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety, Annu Rev Microbiol, 55, 201-234.
27. Scott, T. M., Rose, J. B., Jenkins, T. M., Farrah, S. R. and Lukasik, J., 2002. Microbial source tracking: current methodology and future directions, Appl Environ Microbiol, 68, 5796-5803.
28. Ishii, S., Yan, T., Shively, D. A., Byappanahalli, M. N., Whitman, R. L. and Sadowsky, M. J., 2006. Cladophora (Chlorophyta) spp. harbour humanbacterial pathogens in nearshore water of Lake Michigan, Appl Environ Microbiol, 72, 4545–4553.
29. Byappanahalli, M. N., Shively, D. A., Nevers, M. B., Sadowsky, M. J., Whitman, R. L., 2003. Growth and survival of Escherichia coli and Enterococci populations in the macro-alga Cladophora (Chlorophyta). FEMS Microbiol Ecol, 46, 203–11.
30. Beuchat, L. R., 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables, Microbes Infect, 4, 413–423.
31. Fish, J. T., Pettibone, G. W., 1995. Influence of freshwater sediment on the survival of Escherichia coli and Salmonella sp. as measured by three methods of enumeration, Lett Appl Microbiol, 20, 277–281.
32. Crump, B. C., Baross, J. A., 1996. Particle-attached bacteria and heterotrophic plankton associated with the Columbia River estuarine turbidity maxima, Mar Ecol Prog Ser, 138, 265–273.