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Identification, Detection and Enumeration of *Klebsiella* based on Culture-Independent Real-Time PCR: "The *Klebsiella*: 'An Emerging Environmental Threats' in Riverine-Systems"

Chandra Bali Patel[1,2,*], Ram Sanmukh Upadhyay[2]

[1]Department of Botany, Rastriya Kisan (PG) College, Shamli-247776 UP, India

[2]Department of Botany, Banaras Hindu University, Varanasi-221005, UP, India

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ABSTRACT

Klebsiella is one of the fecal coliform bacteria of family *Enterobacteriaceae.* It is an opportunistic pathogen which previously used as an indicator bacterium along with fecal coliforms namely *Escherichia coli, Citrobacter*, and *Enterobacte*r, but is now known to cause various diseases in human beings. In present study we collected 126 samples from riverine-systems and confirmed the existence of *Klebsiella* using the conserved sequence of *mdh* gene with the application of SYBR green real-time PCR, which demonstrated very high specificity and stringency in detection strategy. The *Klebsiella* levels in surface water were 8.28 x10^2 ±17, 4.76 x10^3 ±122, 6.0 x10^3±178, 9.96 x10^3 ±315, 1.27 x10^4 ±463, 2.76 x10^4 ±1085, cfu/100ml; associated with hydrophytes were 5.80 x10^4 ±2320, 2.48x10^4±806, 2.86x10^4 ±963, 1.01x10^4 ±249, 2.62x10^4 ±830, 2.46x10^4 ±706 cfu/10g and in sediments were in the range of 1.89 x10^4 ±408, 2.46 x10^4 ±530, 3.20 x10^4 ±1004, 2.71 x10^4 ±777, 2.82 x10^4 ±693 and 8.88 x10^4 ±3214 cfu/10 g ($p<0.05$) at sampling site # 1, site # 2, site # 3, site # 4, site # 5 and, site # 6, respectively. This assay could be applied in the detection and monitoring of environmental samples to check waterborne diseases and *Klebsiella* caused outbreaks.

Corresponding Author:
Chandra Bali Patel

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

1. INTRODUCTION

The changing global environments, especially aquatic environment has led to an imbalanced abundance and distribution of microbiota which resulted into emergence and re-emergence of microbial pathogens. Surface and potable waters are heavily contaminated with microorganisms leading to ranges of water-borne diseases. Hence, the microbiological assessment is used worldwide to monitor and control the quality for safety of water. Many potential pathogens could be associated with water and thus it is impractical to screen samples for all probable pathogens. Hence the indicator microorganisms are considered necessary to track the potential pathogens in the aquatic environment. The guidelines and regulations for safe recreational and potable water require determination of absence of 'indicator' microorganisms [26]. The most widely used indicator microbial system is 'coliform bacteria'. These bacteria usually survive in the intestinal tract of homeo-thermals and are discharged through fecal matter. In addition, most coliform bacteria can also exist widely in natural environments including soil, surface water, and to some extent in groundwater [7]. Fecal coliforms are the most common bacterial pollutant in rivers and streams [10]. Non-pathogenic coliforms can survive in water in more adverse conditions as compared to other pathogenic bacteria. There are four genera of fecal coliforms namely *Escherichia, Klebsiella, Enterobacter* and *Citrobacter*. *Klebsiella* is Gram negative, rod-shaped bacteria belong to the family *Enterobacteriaceae*. *Klebsiella* is an important opportunistic pathogen and causes various diseases in human beings such as urinary tract infection, wound infection, septicemia, diarrhea, respiratory tract infection [19] liver abscess [15]. The unbiased and inappropriate use of antibiotics resulted into gain of resistance against them in *Klebsiella*; hence, multidrug resistance (MDR) imposed severe health risks to humans and animals [9]. River Gomti traverses through a very populous city Lucknow (latitude, 26.28 N; longitude, 80.24 E) the state capital of Uttar Pradesh, and it meet the supply of about half of the total domestic water (about 550 mLd) of the town area. In spite of this water demand, Gomti is one of the

most polluted rivers due to huge amount of domestic waste water supply into it which led to eutrophication and bacterial growth. Hence it is crucial to detect *Klebsiella* in the environment to judge and monitor the outbreaks. In present study we have detected *Klebsiella* from riverine-systems such as surface water, hydrophytes and sediments through fast and reliable detection method targeting *mdh* (malate dehydrogenase) gene and employing DNA intercalating dye SYBR Green [17] probe based quantitative PCR (q-PCR) assay.

2. MATERIALS AND METHODS

2.1 Designing of primers specific to *mdh* gene

The complete coding sequences of *mdh* gene (Accession Numbers: FJ483678, FJ483679, FJ483680, FJ483681, FJ483683, FJ483684, FJ483685, FJ483686, AY367377, FJ483693, FJ483691, FJ483692, AY367378, AY367379, AY367380) were retrieved from NCBI GenBank database (http://www.ncbi.nlm.nih.gov) to design a set of primers for specific detection of *Klebsiella* in environmental samples. The alignment of the sequences retrieved for *mdh* gene were created using ClustalW program (www.ebi.ac.uk/clustalW) to find out the conserved sequences. A set of primers for *mdh* gene was designed in highly conserved region of the gene using Beacon Designer 5.0, Premier Biosoft International (Table 1). The specificity of primers was checked against known microbial genomes and sequences by BLAST (Basic Local Alignment Search Tool) program (http://www. ncbi.nlm.nih.gov/BLAST/) to certify homology-free with known gene sequences of other water-borne pathogens. Entire primers used in present study were synthesized from Metabion (Gmbh, Germany).

Table 1. Sequence of primers were used to detect *Klebsiella* in environmental samples

Name of the gene	Primers Sequences (5'-3') **F**: Forward; **R**: Reverse	Length (nt)	Tm (°C)	% GC	Product Length (bp)
mdh	**F:** CGCGTAAGCCCGGCATGGAT	20	59.9	65	276
	R: GGACTTCCACCTCGGTTGCCG	21	59.7	66.7	

2.2. Generation of standard curve for quantitative enumeration of *Klebsiella* spp.

To prepare the standard curve (Figure 2), reference strain (*Klebsiella* MTCC 618 carrying *mdh* gene procured from Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology (IMTECH), Chandigarh, India) was cultured in triplicate in 15 milliliter Luria–Bertani (LB) broth for overnight at 37± 1°C to nearly optical density of 0.8 at 600 nm. The numbers of colony forming units (CFU/ml) of cell suspension was calculated by plating 100 µl of the three-fold diluted culture onto five Luria–Bertani agar plates. The average number of CFU 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 ten-fold serially diluted 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 [12] and qPCR assays for *mdh* gene was performed using an iCycler (BIO-RAD, USA) real-time PCR instrument and Quantifast SYBR Green PCR kit (Qiagen, Germany). Briefly, the reaction mixture constituted 0.2 µM of the forward and reverse oligo-nucleotide primer (1 µl each) for *mdh* 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. In negative controls 5 µl sterilized Milli-Q water used as template. The PCR amplification protocol for the assay *mdh* gene consisted of initial denaturation of 5 min at 95 °C, followed by 45 cycles of three steps consisting of 10s at 95 °C, 20s at 55.8 °C and 20 s at 72 °C. The fluorescence signals were measured at the end of each extension step. At the completion of PCR amplification, 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 /S. 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). The qPCR assays to generate standard curves for *mdh* gene 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 *Klebsiella* in Riverine-Systems

2.3.1 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 1). This river traverses a distance of about 730 km in the Indo-Gangetic alluvial region before its confluence with river Ganga. The river contains water throughout the year and exhibits sluggish flow except in the monsoon season. In this study, six sampling sites were selected in up-to-downstream along the river Gomti namely Ghaila-Bridge (site# 1, located in upper-most stream of river from where river enters the Lucknow city), Gau-Ghat (site# 2, cloth washing and bathing spot), Shaheed Smarak (site# 3, bathing ghat and holy spot), Bhaisakund (site# 4, human cremation spots), Chandiamau (site# 5, bathing and human activities) and Indira Jal Setu (site# 6, down-most stream location in the landscape) were selected

on the basis of various human activities on the banks of river Gomti. A cross-sectional approach was used to collect river water samples. Samples were collected in triplicate (n = 54) from six locations situated in up-to-down-gradient fashion. In brief, three transects were established randomly at each site and one liter water samples were collected 30 cm below water surface from left, mid and right banks of the river along each transect. Water samples were stored in sterile glass bottles, labeled and transported on ice to the laboratory for analysis. Sample processing and analysis were conducted within 6 h after sample collection. DNA isolation was carried out by boiling preparation method. The quantity and quality of extracted DNA was measured by NanoDrop spectrophotometer ND 3.0 1000 (NanoDrop Technologies, Wilmington, DE, USA).

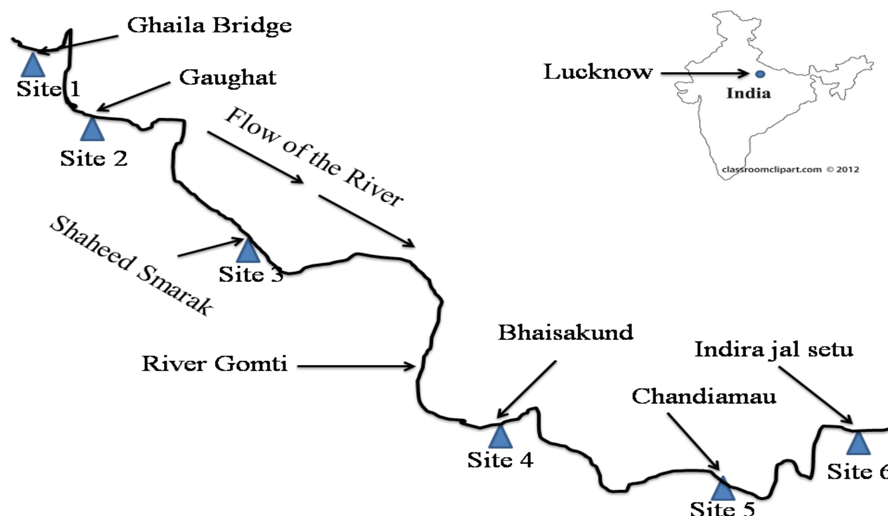

Figure 1. Map of sampling sites of surface water, plants and sediment in river Gomti in Lucknow city. The figure was taken and redrawn from Map of India-Google map (map not to scale).

2.3.2. Collection of Hydrophytes from Riverine System and DNA Isolation

Same sampling sites (Figure 1) were selected and three transects were made randomly to collect hydrophytes. The submerged hydrophytes *Potamogeton crispus* (L.) were taken into consideration. The samples were collected as per APHA protocol [1]. Along each transect, 1 m^2 quadrats were set at a distance of 0.5 m from left and right banks. All the *P. crispus* (n=6) from each sampling sites within the quadrats were collected and pooled in sterile zip-lock bags. One liter water surrounding to hydrophytes were also collected in sterilized bottles along each transect at respective sites. Further, the plant associated microbiota was recovered in 500 mL phosphate buffered saline and it was pooled with 500 ml plant surrounding water. A 10 and 100-fold diluted portion of this sample was used for estimation *Klebsiella* 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 plant followed by three sequential rinsing provided an average recovery of 95% of the bacteria from the 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 and measured the quantity and quality of extracted DNA.

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

Same sampling sites were selected for collection of sediments in river Gomti (Figure 1). Sediment core samples (~250 g) were collected directly from an approximately 0.5 cm depth of the sediment surface at three different positions (~1m distance) of each sampling sites from both banks of river using a sterile stainless-steel spatula and placed in plastic bags. All the samples (n=6) from each sampling site were mixed and prepared the grab sample. Finally, 10 ± 0.5 g fresh weights from each site were added to 100 ml of sterile phosphate-buffered saline. After vortexing, the samples were kept in incubator shaker (INNOVA 4230) at 220 rpm at 37 ^{0}C for 10 minutes and then allowed to gravity settle at the bottom of the tube. The supernatant which mostly contain bacteria were then carefully removed for isolation of multi-genomic DNA using boiling lysis followed by precipitation by sodium acetate-ethanol method. The quantity and quality of extracted DNA was measured by Nano Drop spectrophotometer ND 3.0 1000 (Nano Drop Technologies, Wilmington, DE, USA).

2.4 Statistical Analyses

For 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 $E = (10^{-1/slope})-1$. A reaction with theoretical 100% efficiency will generate a slope of -3.322. Data obtained from conventional and qPCR were compared by Wilcoxon Rank-SumTest. *Klebsiella* spp. retrieved in this study at different sites was analyzed using one way analysis of variance [8].

3. RESULTS AND DISCUSSION

The present study is based on q-PCR (Real-Time PCR) assay targeting *mdh* gene to detect *Klebsiella* quantitatively in various samples collected from river Gomti in Lucknow. *Klebsiella* loads in riverine-systems were identified in three domains of the river (1) surface water, (2) associated with hydrophytes and (3) sediments. The detection limit of this q-PCR assay was up to 1 CFU/PCR (Figure 2) with PCR efficiency 99.3 % which exhibit the accuracy and robustness of the technique and protocol. A significant variation in, *Klebsiella* contamination at different sites were observed (one way ANOVA, $p<0.05$).

Surface water is the potent source of microorganisms since it carries dissolved as well as suspended organic and inorganic nutrients. Members of family *Enterobacteriaceae* namely *Escherichia, Enterobacter, Citrobacter,* and *Klebsiella* were the most commonly encountered genera in shell egg processing plants [16] and the effluents from various kinds of food processing industries may lead to the discharge of its waste materials into riverine-systems. Additionally, various point and non-point sources of pollution enhance the loads of bacterial population in the river. Some of the microorganisms get settled down to the sediments, some get adhered to hydrophytes, while remaining gets suspended in water bodies. The *Klebsiella* levels in surface water (n=54) were $8.28 x10^2$ ±17, $4.76 x10^3$ ±122, $6.0 x10^3$±178, $9.96 x10^3$ ±315, $1.27 x10^4$ ±463 and $2.76 x10^4$ ±1085, CFU/100ml at sampling site # 1, site # 2, site # 3, site # 4, site # 5 and, site # 6, respectively (Table 2). Analysis for fecal-indicator bacteria by MPN (most probable number) needs 48 h to confirm fecal pollution in a sample and provides no insight about the coliform bacteria [20,26] but this method is quite fast and stringent. Further, it has been reported that a value of total coliform in an environmental sample consists of *E. coli, Citrobacter/Enterobacter* and *Klebsiella* in the ratio 94:4:2 [5,14]. 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 (Data not shown).

Table 2. *Klebsiella* counts in river Gomti in surface water (per 100ml), hydrophytes (per 10g) and sediments (per 10 g)

Sampling sites	Sites Name	Surface water	Hydrophytes	Sediments
Site # 1	GhailaBridge	$8.28 x10^2$±17	$5.80 x10^4$ ±2320	$1.89 x10^4$ ±408
Site # 2	Gaughat	$4.76 x10^3$ ±122	$2.48x10^4$±806	$2.46 x10^4$ ±530
Site # 3	Shaheed Smarak	$6.0 x10^3$±178	$2.86x10^4$ ±963	$3.20 x10^4$ ±1004
Site # 4	Bhaisakund	$9.96 x10^3$ ±315	$1.01x10^4$ ±249	$2.71 x10^4$ ±777
Site # 5	Chandiamau	$1.27 x10^4$ ±463	$2.62x10^4$ ±830	$2.82 x10^4$ ±693
Site # 6	Indira Jal Setu	$8.16 x10^4$ ±3443	$2.46x10^4$ ±706	$8.88 x10^4$ ±3214

Hydrophytes are the important stationary/static habitats of bacteria [2,3,11] in river water since bacteria get attached to plants parts. Bacteria adhering to hydrophytes could be released frequently into water-stream due to wave action along the banks or by anthropogenic activities, and could float several miles downstream along the course of the river. Aquatic flora may be an important and significant nonpoint source of pollution[21]**.** The levels of *Klebsiella* contamination associated with hydrophytes (n=36) were $5.80 x10^4$ ±2320, $2.48x10^4$±806, $2.86x10^4$ ±963, $1.01x10^4$ ±249, $2.62x10^4$ ±830, and $2.46x10^4$ ±706 CFU/10g at sampling site # 1, site # 2, site # 3, site # 4, site # 5 and, site # 6, respectively (Table 2).

The sediments are the silent reservoir of microorganisms in the rivers [4,6]. The heavy particles that cannot float easily in water get settled down at the river bed along with undissolved pollutant and microorganisms hence the sediments can also be one of the important factors which can be helpful in microbial source tracking. The *Klebsiella* were found in sediments (n=36) in the range of $1.89 x10^4$ ±408, $2.46 x10^4$ ±530, $3.20 x10^4$ ±1004, $2.71 x10^4$ ±777, $2.82 x10^4$ ±693 and $8.88 x10^4$ ±3214 CFU/10g at sampling site # 1, site # 2, site # 3, site # 4, site # 5 and, site # 6, respectively (Table 2). 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) [23] and may lead to change in the ecosystem of bottom of the river.

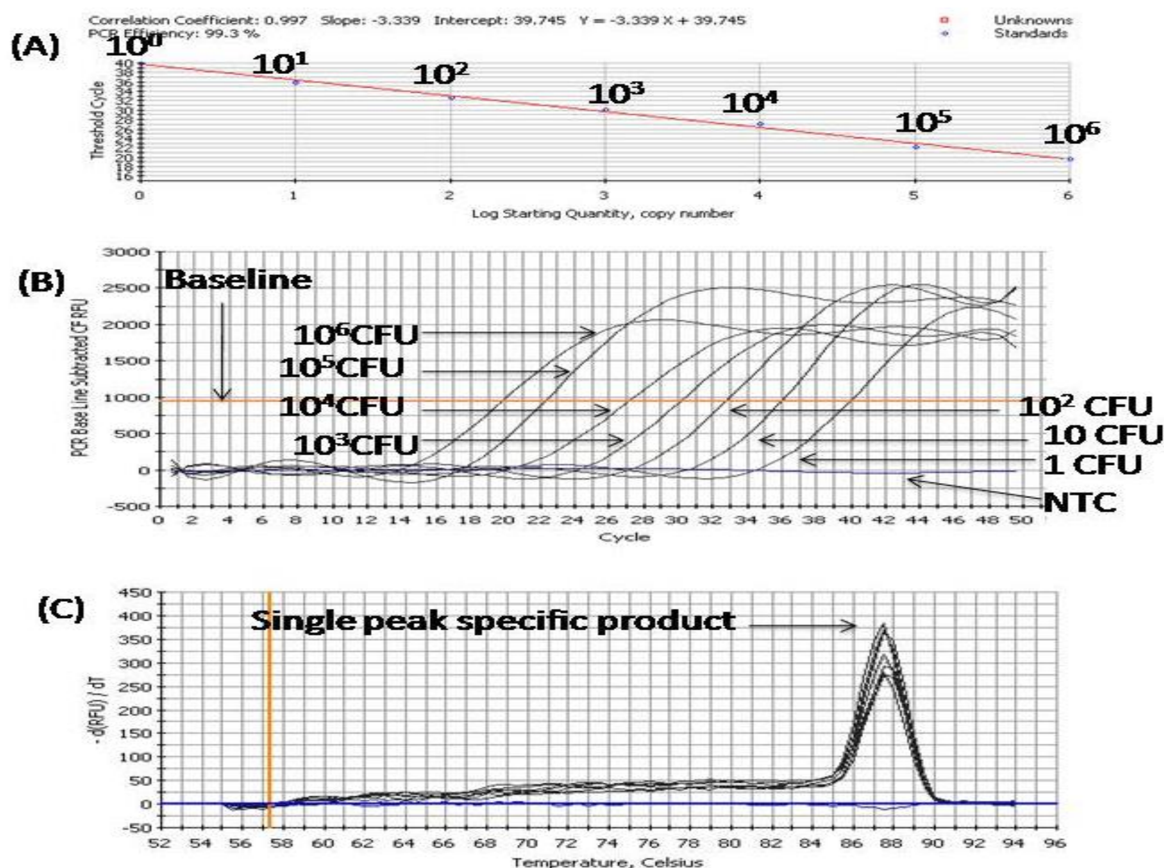

Figure 2. PCR profiling and melting curve analysis of qPCR (A-C). A: Standard curve, B: Amplification curve, C: Melt curve. Product (length 276 bp) amplified from *mdh* gene of 10-fold serially diluted culture of *Klebsiella* species.

The natural water reservoirs have capacity to balance the ecosystem through solar inactivation of pathogens by UV-B radiation and reactive oxygen species (ROS) with external sensitizer molecules[13]. In rivers the survival and die-offs of these biological contaminants are also governed through grazing and predation at various trophic levels [18,22,24]. The detection of *Klebsiella* in river water, sediment and hydrophytes is important to study their role in dissemination and persistence of antimicrobial resistance [25].

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