



# A Multifaceted Microorganism *Klebsiella* And Its Culture-Independent Real-Time PCR Based Detection and Enumeration: 'An Emerging Threats' in Potable Water

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| ARTICLE INFO                                                                                                            | ABSTRACT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
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| <b>Published Online:</b><br>28 February 2026                                                                            | The altered worldwide surroundings and changed microbial population has sternly declined the worth of water quality and executed amplified health risks on human beings due to absence of safe and unspoiled water. <i>Klebsiella</i> is one of the fecal coliform bacteria of family <i>Enterobacteriaceae</i> . It is an opportunistic pathogen which previously used as an indicator for microbial water pollution along with fecal coliforms namely <i>Escherichia coli</i> , <i>Citrobacter</i> , and <i>Enterobacter</i> , but is now known to cause numerous diseases in human beings. In this study we collected 30 samples of potable water and confirmed the existence of <i>Klebsiella</i> using the conserved sequence of <i>mdh</i> gene with the application of SYBR green based real-time PCR, which demonstrated very high specificity and stringency in detection approach. The <i>Klebsiella</i> levels in potable water at sites: A-J targeting <i>mdh</i> gene were $4760 \pm 184.68$ , ND (not detected), ND, ND, $1.52 \times 10^2 \pm 4.51$ , $5.66 \times 10^2 \pm 16.92$ , $5.82 \times 10^2 \pm 18.79$ , $1.94 \times 10^3 \pm 64.80$ , $2.56 \times 10^3 \pm 89.08$ and $3.72 \times 10^3 \pm 148.06$ CFU/100 ml, ( $p < 0.05$ ) respectively. This assay could be applied in the detection and regular monitoring of potable water to check waterborne diseases and <i>Klebsiella</i> caused outbreaks. |
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| <b>KEYWORDS:</b> fecal coliforms, bacteriological contamination, real-time PCR, <i>Klebsiella</i> , pathogenic bacteria |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |

## 1. INTRODUCTION

The altering worldwide milieus, particularly water environment has led to an inappropriate abundance and distribution of microbiota which resulted into emergence and re-emergence of microbial pathogens. Hence, the availability of safe and pristine potable water becomes a global challenge. Surface and potable waters are heavily contaminated with microorganisms leading to assortments of water-borne diseases. Hence, the microbiological assessment is used worldwide to monitor and control the quality and 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 (WHO, 2002). The most widely used indicator microbial system is 'coliform bacteria'. These bacteria usually subsist 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 (Francy *et al.*, 2000). Fecal coliforms are the most common bacterial pollutant in rivers and streams (Hill *et al.*, 2006). Non-pathogenic coliforms can survive in water in 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*. In this study we have detected the *Klebsiella* spp. in the environmental samples of potable water by targeting *mdh* (malate dehydrogenase) gene by SYBR Green based quantitative PCR (q-PCR) assay.

*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 (Podschi and Ullmann, 1998), liver abscess (Lederman and Crum, 2005). 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 (Habeeb Khadri, *et al.*, 2007; Soler Bistue, *et al.*, 2008).

River Gomti, a major tributary of river Ganga, is the life-line of 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, as it meets about half of the total domestic water demand (about 550 mLD) of the town (Singh *et al.*, 2004). However, river Gomti is identified as one of the most polluted rivers in India and contain huge amount of organic and inorganic substances leading to eutrophication of the river water (Singh *et al.*, 2004) and hence flourish bacteria including *Klebsiella* (Patel *et al.* 2024).

Hence it is utmost essential to detect *Klebsiella* in the water samples to assess and monitor the outbreaks. The present study has demonstrated the fast and reliable detection method targeting *mdh* gene and DNA intercalating dye SYBR Green (Patel *et al.*, 2016) probe to detect *Klebsiella* from potable water samples.

## 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 targeting *mdh* gene.

A set of primers for *mdh* gene was adopted from Patel *et al.*, 2024. In brief, the alignment of the sequences retrieved for *mdh* gene were created using ClustalW program ([www.ebi.ac.uk/clustalW](http://www.ebi.ac.uk/clustalW)) to determine 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 determined against known microbial genomes and sequences by BLAST (Basic Local Alignment Search Tool) program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to ensure no homology was observed with known gene sequences of other water-borne pathogens. All the primers used in this study were synthesized from Metabion (GmbH, Germany).

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

For the preparation of standard curve (Figure 2), reference strain (*Klebsiella* MTCC 618 exhibiting *mdh* gene procured from Microbial Type Culture Collection (MTCC) at

Institute of Microbial Technology (IMTECH), Chandigarh, India) was grown in triplicate in 15 ml Luria–Bertani (LB) broth for 12h 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 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 serially diluted ten-fold to yield 10<sup>7</sup> 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) 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 contained 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<sup>6</sup>–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 (T<sub>m</sub>) 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. Fluorescence was monitored continuously to confirm amplification specificity. The T<sub>m</sub> 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<sup>2</sup> values were calculated to check the reproducibility of the assays.

### 2.3 Quantitative enumeration of *Klebsiella* in potable water

For potable water supply in Lucknow city, water is pumped from the river Gomti at Gaughat, which is outside the city, and is sent through a pipeline to Lucknow Jal Sansthan, Aishbagh, 4 km away, where the water is purified by alum treatment, filtration, and chlorination before being released into the drinking water supply (Ram *et al.*, 2008 b; Clever *et al.* 2000; Shrivastava *et al.* 2004). To test the possibility of the contamination of potable waters by *Klebsiella* due to defective water distribution systems and insufficient treatment during production, one liter water samples were collected in triplicate for culture free quantitative enumeration at ten sites. Site: A,

Gaughat (raw water intake point in the river Gomti) site : B, Aishbagh waterworks (before water enters the 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, MotiNagar (pipeline that percolated and ran along open drainage); site : J, KGMU (Figure 1). All the potable water samples were collected into sterile glass bottles on the same day in densely populated areas across the urban boundaries of Lucknow city, stored on ice, and transported to the laboratory for analyses within 6 h (APHA, 1998). The sites were numbered in order of the sample collection. Bacteriological quality of the potable water samples collected in this study was assayed to ascertain *Klebsiella* contamination. The quantity and quality of extracted DNA was measured by Nano Drop spectrophotometer ND 3.0 1000 (Nano Drop Technologies, Wilmington, DE, USA).

Multigenomic DNA from water (500 mL) was extracted by boiling preparation method as per procedure described by Jyoti et al. (2010) and quantification of *Klebsiella* based on *mdh* gene was done by ten-fold standard curve was generated by diluting the culture of *Klebsiella* MTCC 618.

#### 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<sup>TM</sup> Real-Time Detection System Software Version 3.0. Amplification efficiency (*E*) was estimated by using the slope of the standard curve and the formula  $E = (10^{-1/\text{slope}}) - 1$ . A reaction with theoretical 100% efficiency will generate a slope of -3.322. Data obtained from conventional and qPCR were compared by Wilcoxon Rank-Sum Test. *Klebsiella* spp. retrieved in this study at different sites was analyzed using one way analysis of variance (Gomez and Gomez, 1984).

### 3. RESULTS

The SYBR Green based qPCR assays targeting *mdh* gene were applied to detect *Klebsiella* quantitatively in potable water samples collected from Lucknow. A significant variation in, *Klebsiella* contamination at different sites were observed (one way ANOVA,  $p < 0.05$ ). Potable water samples collected from sites: E-J located in water distribution system that either percolated or ran along open drainage, exhibited the presence of *Klebsiella* (Table 2). The *Klebsiella* levels at sites: A-J targeting *mdh* gene were  $4760 \pm 184.68$ , ND (not detected), ND, ND,  $1.52 \times 10^2 \pm 4.51$ ,  $5.66 \times 10^2 \pm 16.92$ ,  $5.82 \times 10^2 \pm 18.79$ ,  $1.94 \times 10^3 \pm 64.80$ ,  $2.56 \times 10^3 \pm 89.08$  and  $3.72 \times 10^3 \pm 148.06$  CFU/100 ml, respectively.

### 4. DISCUSSION

The present study is based on q-PCR assay targeting *mdh* gene. 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. The advantage of low limit detection by q-PCR could be applied in monitoring and management of outbreaks caused by *Klebsiella*.

This study was conducted into two realms of the environments. The first realm was potable water. The *Klebsiella* were observed at various sampling sites. Site: A represents surface water whereas, sites: B-J represent potable water. At sampling sites: B, C and D *Klebsiella* were not detected since at these sites the potable water pipe-line supply was neither percolated nor ran along open drainage system. At sampling sites: E-J the *Klebsiella* were detected because at these sites pipe-line supply was either percolated or ran along open drainage systems. Hence, the possession of *Klebsiella* in potable waters at sampling sites: E-J were due to local pollution. The presence of *Klebsiella* in potable waters is becomes the not good sign for human health. When the human being consumes this contaminated water, they intake the bacteria as well, which lead to various types of water borne disease. To cure their illness, it requires to take of medicines including antibiotics. The inappropriate applications of antibiotics in disease control create a selection pressure on bacteria either to kill or survive. Some bacteria may kill but other choose the alternative path to be survive against the antimicrobials. This survival pathway is termed as antibiotic resistance. The existence of *Klebsiella* in the riverine system was also abundant (Patel *et al.*, 2024), hence the monitoring of environment for the betterment of human health becomes necessary.

#### Author Contributions

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

RU: Analyzed as well as interpreted the data.

### ACKNOWLEDGMENTS

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### List of Figures

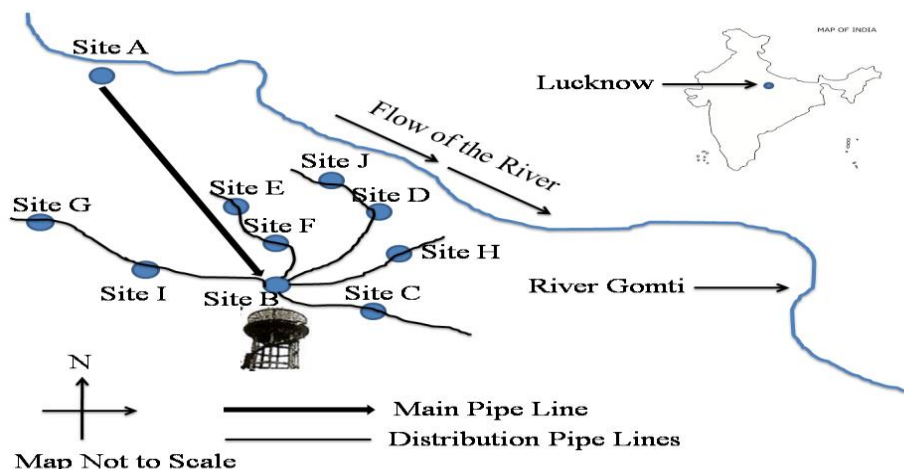


**Figure 1. Sampling sites of potable water in Lucknow city.**

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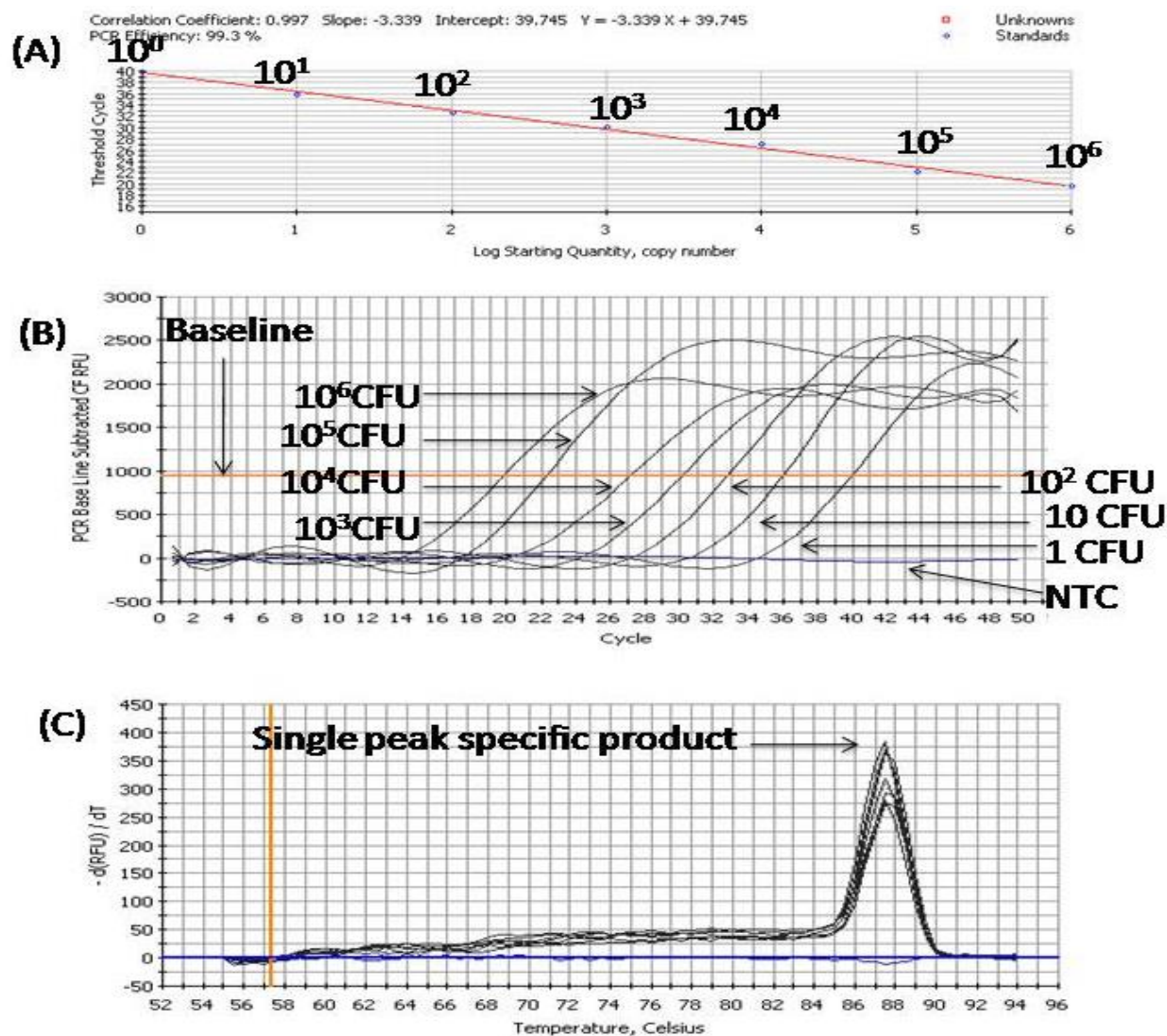


Figure 2. PCR profiling and melting curve analysis of qPCR (A-C). A: Standard curve, B: Amplification curve, C: Melt curve. Product (length 276 bp) amplified from *mdh* gene of 10-fold serially diluted culture of *Klebsiella* species.

List of Tables

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

| Name of the gene | Primers Sequences (5'-3') | Length (nt) | T <sub>m</sub> (°C) | % GC | Product Length (bp) |
|------------------|---------------------------|-------------|---------------------|------|---------------------|
| <i>mdh</i>       | F: CGCGTAA GCCCGGCATGGAT  | 20          | 59.9                | 65   | 276                 |
|                  | R: GGACTTCCA CCTCGGTTGCCG | 21          | 59.7                | 66.7 |                     |

(Adopted from Patel *et al.*, 2024)

Table 2 *Klebsiella* counts in potable water (per 100 ml)

| <sup>a</sup> Sampling Sites | Sites Names  | <i>Klebsiella</i> /100mL <sup>bc</sup> |
|-----------------------------|--------------|----------------------------------------|
| Site: A                     | Gaughat      | 4760± 184.68                           |
| Site: B                     | Aishbagh     | ND <sup>d</sup>                        |
| Site: C                     | Husain Ganj  | ND                                     |
| Site: D                     | Kaiserbagh   | ND                                     |
| Site: E                     | Nakkhas      | 1.52 x10 <sup>2</sup> ± 4.51           |
| Site: F                     | Shastrinagar | 5.66 x10 <sup>2</sup> ±16.92           |

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|         |              |                                |
|---------|--------------|--------------------------------|
| Site: G | Saadatganj   | 5.82 x10 <sup>2</sup> ±18.79   |
| Site: H | City Station | 1.94 x10 <sup>3</sup> ± 64.80  |
| Site: I | Motinagar    | 2.56 x10 <sup>3</sup> ± 89.08  |
| Site: J | KGMU         | 3.72 x10 <sup>3</sup> ± 148.06 |

<sup>a</sup>Site A: represents surface (river) water whereas sites B-J represents potable water

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