Research Paper

Exploration of coliform diversity in drinking water resources by culture-independent approaches

A. Shiva Shanker, Praveen Kumar Vootla and Pavan Kumar Pindi

ABSTRACT

The coliform group has been widely used as an indicator of water quality and has historically led to a public health protection concept. Presence of pathogens in drinking water may raise several health problems in humans from mild illnesses to serious waterborne diseases. In spite of several measures taken, water quality is always a pertinent issue prevailing in diverse water systems. So far, coliform contamination and diversity could not be adequately explored as traditionally used culture-dependent methods have a limited capacity to characterize microbiota from their respective sources. The study was designed for assessment of microbial diversity by culture-independent approaches placing emphasis on exploring the total coliform diversity in two drinking water reservoirs, Raman Pahad and Koilsagar of Mahabubnagar district, Telangana, India. Principal analysis based on 16S rRNA gene clone libraries revealed that Raman Pahad library clones belonged to genus *Enterobacter* (41.5%), followed by *Citrobacter* (25.03%), *Klebsiella* (17.86%), *Escherichia* (12.20%), and the least being *Hafnia* (3.39%). The clones in Koilsagar belonged to genus *Enterobacter* (46.42%) as the most predominant, followed by *Citrobacter* (32.14%) and *Escherichia* (21.42%). Comparatively, *Enterobacter* was observed to be the most predominant (representing 50%) of the total clones in both reservoirs.

Key words | 16S rRNA, coliform, contamination, drinking water, public health, reservoir

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HIGHLIGHTS

- The study was designed for assessment of microbial diversity by culture-independent approaches laying emphasis on exploring the total coliform diversity in two drinking water reservoirs, Raman Pahad and Koilsagar of Mahabubnagar district, Telangana, India.
- Principal analysis based on 16S rRNA gene clone libraries revealed that, Raman Pahad library clones belonged to genus Enterobacter (41.5%), followed by Citrobacter (25.03%), Klebsiella (17.86%), Escherichia (12.20%) and the least being Hafnia (3.39%); whereas the clones in Koilsagar belonged to genus Enterobacter (46.42%) as the most predominant followed by Citrobacter (32.14%) and Escherichia (21.42%).
- Comparatively, Enterobacter was observed to be the most predominant (representing 10%) of the total clones in both reservoirs.

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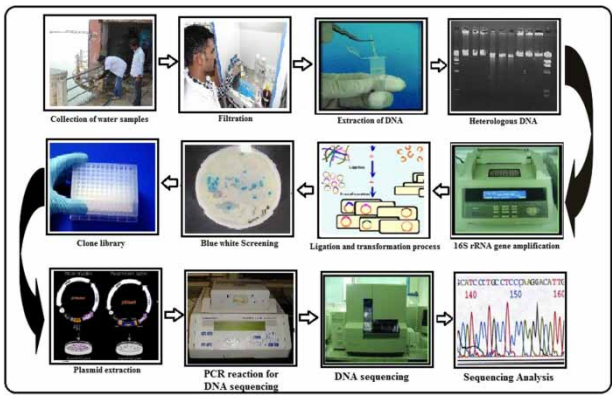
GRAPHICAL ABSTRACT



INTRODUCTION

Water is a pertinent component of life and its main sources include groundwater, surface and rain water. Potable water is defined as having a satisfactory quality in terms of its physical, chemical, and bacteriological parameters so that it can be securely used for drinking and cooking (Jain *et al.* 2010). It is reported that 91% of the global population uses improved water sources which is an increase from 76% in 1990 (Dos Santos *et al.* 2017). The most common and widespread health risks associated with drinking water in developing countries are due to the presence of various microbes (Shakoor *et al.* 2018), and approximately 663 million people continue to lack access to improved drinking water sources (WHO/UNICEF 2015). In 2011, the WHO reported that few opportunistic bacteria along with enteric pathogens persevere in the environment, the presence of which could be unsafe to the elderly, infants, and immunosuppressed individuals. The WHO/UNICEF Joint Monitoring Programme (JMP) for Water Supply, Sanitation and Hygiene presents updated national, regional, and global estimates for water, sanitation, and hygiene (WASH) in households in its 2019 update report, stating that the population using safely managed drinking water services has increased from 61 to 71%. Safely managed sanitation services have increased from 28 to 45 and 60% of the global population has basic hand washing facilities with soap and water at home.

Acute microbial diarrheal diseases are a major public health problem in developing countries as these countries have the lowest financial resources and poorest hygiene facilities. Among all age groups, children under the age of five are the most affected by microbial diseases transmitted through water (Seas *et al.* 2000; Vetrimurugan *et al.* 2013). Some of the diseases that are transmitted through contaminated drinking water are typhoid, cholera, infectious hepatitis, and disease caused by *Shigella* spp and *Escherichia coli* O157 (Lenart-Boroń *et al.* 2017).

Given that abundant pathogens occur in feces, water is therefore monitored for microbial contamination using indicator organisms such as total coliforms and *E. coli* (Stelma & Wymer 2012). Total coliforms are frequently encountered in the aquatic environment, in soil, vegetation, and universally in huge numbers in the feces of warm-blooded animals (Cabral 2010). An important subgroup of this collection is the fecal coliform bacteria, the main member being *E. coli*. These organisms differ from the total coliform group by their ability to grow at higher temperatures. Insufficient treatment of surface waters, defective water distribution pipelines of the drinking water supply, and faulty sewage collection systems have led to contamination of potable water by enterohemorrhagic *E. coli* (EHEC) and other pathogenic bacteria (Ram *et al.* 2008, 2011). *E. coli*, thermotolerant coliforms, and/or intestinal

enterococci are considered as main fecal indicator microbes for testing the quality of water (Van der Wielen & Medema 2010).

Bacterial diversity surveys of natural waters are significant approaches currently employed to assess the relation between various enteric bacteria, to support management policies or to sustain risk assessment studies (Molina *et al.* 2015). Culture-dependent methods have been constantly used to optimize, identify, and quantify the presence of organisms relevant in terms of quality control and public health (Leclerc & Moreau 2002; Mossel & Struijk 2004). These methods reveal the enormous diversity of uncultured organisms, and thus, highlight the call for alternative approaches for the analysis of water bacterial diversity (Amann *et al.* 1995; Alain & Querellou 2009). A number of scientific and technological developments, such as molecular fingerprinting (Dewettinck *et al.* 2001), metagenomic and gene library (Cottrell *et al.* 2005), PCR-DGGE (Wu *et al.* 2006) and FISH (Bottari *et al.* 2006), but above all, the inexpensiveness of the nucleic acid sequencing methods, have brought noticeable improvements to bacterial diversity studies. 16S rRNA gene clone libraries, fluorescence *in situ* hybridization (FISH) or denaturing gradient gel electrophoresis (DGGE) are some of the methods used nowadays to explore the bacterial diversity in waters (Lyautey *et al.* 2005; Piterina & Pembroke 2013; Shanker *et al.* 2019). More recently, the potential of the high-throughput 454 pyrosequencing to explore the environmental diversity has been emphasized (Bae *et al.* 2019). The use of culture-independent approaches to complement culture-dependent methods is much preferred for inferring the significance of a specific taxonomic group in the entire community (Shivaji *et al.* 2011).

In view of the above-mentioned facts and witnessing the routine water-borne outbreaks in and around Mahabubnagar district, an attempt was made to decipher the actual problem for several diarrheal outbreaks with the most reliable and advanced molecular methods. Uthappa *et al.* (2015) reported that several diarrheal outbreaks observed on a frequent basis may be attributed to the poor quality of potable water supplied mainly from the reservoirs in the Mahabubnagar district. Therefore, the work reported herein was designed to assess the total coliforms and bacterial diversity of two water reservoirs of Mahabubnagar, Telangana, India by 16sRNA sequencing.

MATERIALS AND METHODS

This study was carried out from March, 2017 to February, 2018 in the combined/erstwhile Mahabubnagar district of Telangana State, India. Sampling locations selected for the above study were Koilsagar village of Deverakadra Mandal in Mahabubnagar district and Kothakota mandal of Wanaparthy district. Both the sampling sources were water reservoirs, i.e., Raman Pahad balancing reservoir (coordinates: 16°22′04″N latitude 77°52′20″E longitude) shown in Figure 1(a) and Koil Sagar reservoir (coordinates: 16°44′ N latitude and 77°45′E longitudes) shown in Figure 1(b).

Sample collection

The drinking water samples were collected at four points, namely, north, east, west and south of each reservoir in sterile bottles at a depth of about 30 cm with the bottle's mouth facing the current and ensuring that the water entering the bottle had no contact with the hand and air. The utmost care was taken to avoid contamination of water with atmospheric bacteria. The sample bottles were capped and labeled appropriately with all necessary details such as source, time, and date of collection. Samples were stored at 4 °C and shipped to the laboratory. All the samples were initially passed through membrane filters of 0.45 μm in order to trap bacteria and were assessed within 6 hours of collection for their bacteriological quality using molecular studies.

Determination of bacterial diversity by culture-independent method

Extraction of total DNA from water and PCR amplification of the 16S rRNA gene

Total DNA was isolated from the drinking samples essentially according to the methods described earlier. Primers 1492r primer (5′-TACCTTGTTACGACTT) and 27f (50-GGC GGTGTG TAC AAG GCC C-30) were used to amplify 1.5 kb 16S rRNA gene. Amplification was done following a method reported by Shivaji *et al.* (2009). The polymerase chain reaction (PCR) amplicon was electrophoresed on a 1.0% agarose gel and visualized following staining with ethidium bromide (0.3 μg/mL). The PCR product was purified

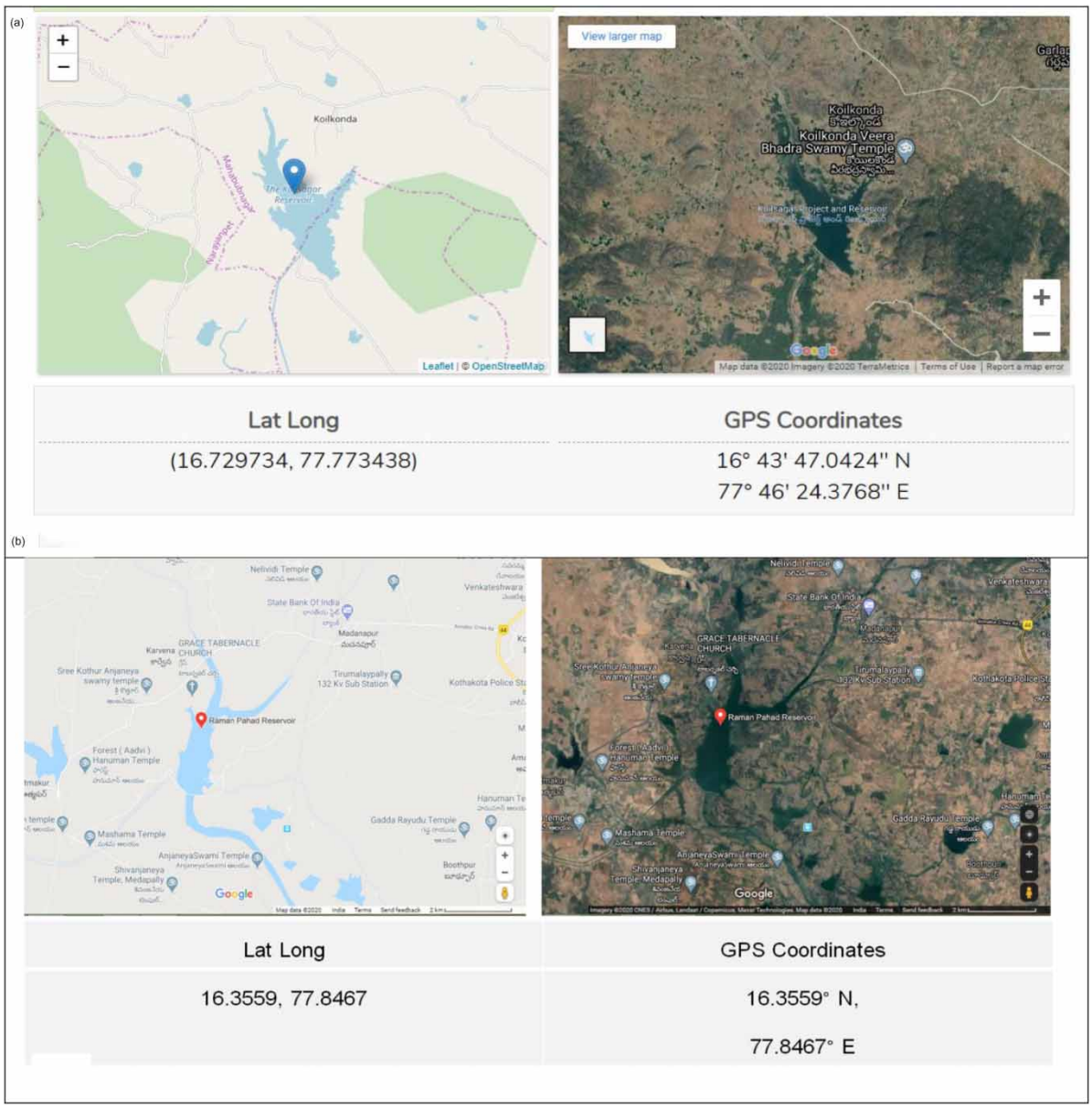


Figure 1 | Location of (a) Koil Sagar reservoir and (b) Raman Pahad reservoir.

with the Quia quick PCR purification kit (Qiagen Inc, Chatsworth, USA) according to the instructions provided.

16S rRNA gene cloning and library construction

The purified PCR product obtained earlier was cloned into pMOS Blue Blunt End vector system (Amersham Biosciences, New Jersey, USA) following the instructions of the manual. Transformants were selected on a LB agar plate containing 40 μg/mL X-gal and 12.5 μg/mL ampicillin and incubated at 37 °C overnight. Clones were maintained on LB agar plates containing X-gal and ampicillin.

16S rRNA gene sequencing and phylogenetic analysis

For 16S rRNA gene sequencing, DNA was prepared using the Mo Bio microbial DNA isolation kit (Mo Bio Laboratories Inc., Solano Beach, CA, USA) and sequenced as

described previously (Lane 1991). The resultant, almost complete sequence of the 16S rRNA gene contained 1,502 nucleotides. The 16S rRNA gene sequence of the isolate was subjected to BLAST sequence similarity search and EzTaxon to identify the nearest taxa. The entire related 16S rRNA gene sequences were downloaded from the database (http://www.ncbi.nlm.nih.gov), aligned using the CLUSTAL_X program and the alignment was corrected manually. Phylogenetic tree was constructed using tree-making algorithm and the maximum likelihood (ML) using the PhyML program.

Nucleotide sequence accession numbers

The representative 16S rRNA gene clone library in this study was deposited in the GenBank database. The accession numbers for the clones of Raman Pahad were KR612007-KR612060 and for Koilasagar were KR612049-KR612071.

RESULTS AND DISCUSSION

Analysis of coliform bacterial diversity

The water samples collected from the Raman Pahad and Koilsagar water reservoirs were monitored for total coliforms and *E. coli*. The study revealed the presence of fecal colifrom, i.e., *E. coli* in RP and KS, respectively, of 12.20 and 21.42%. Whereas total coliforms were in the ratio *Enterobacter*:*Citrobacter*:*Klebsiella*:*Escherichia*:*Hafnia* of 41:25:17:12:3 for RP reservoir and *Enterobacter*:*Citrobacter*:*Escherichia* in 46:32:21 for KS. Enumeration of this population in the microbial aquatic ecosystem has been universally applied to certify the sanitary quality of water. The samples yielded about 30–80 μg DNA per 100 mL concentrated water. About 200 ng of DNA was used for constructing a 16S rRNA gene library. The libraries constructed from DNA isolated from Raman Pahad and Koilsagar samples consisted of 785 and 323 clones, respectively, with an insert size of approximately 1 kb. The affiliation of each and every clone to the nearest phylogenetic neighbor is based on 16S rRNA gene sequence. Thus, 16S rRNA gene could prove to be a constructive diagnostic tool for identifying the presence of pathogens in water samples (Srinivasan *et al.* 2015).

Diversity within clone libraries

BLAST sequence similarity analysis of the clone libraries of Raman Pahad and Koilsagar indicated that clones belonged to the phylum Proteobacteria (Figures 2 and 3). Of the 785 clones examined in the Raman Pahad library, the predominant sequences were from the genus *Enterobacter* (41.5%), followed by *Citrobacter* (25.03%), *Klebsiella* (17.86%), *Escherichia* (12.20%), and the least being *Hafnia* (3.39%); whereas among the 323 clones in Koilsagar the genus *Enterobacter* (46.42%) was prevalent followed by *Citrobacter* (32.14%) and *Escherichia* (21.42%). Dufour (1977) and Leclerc *et al.* (2001) reported *E. coli*:*Citrobacter*/*Enterobacter*:*Klebsiella* in the ratio of 94:4:2 in natural habitats. According to Patel *et al.* (2016), fecal coliforms were in the ratio of 88.89:4.78:5.11:1.52 for *E. coli*:*Enterobacter*:*Citrobacter*:*Klebsiella*, respectively. Earlier reports also proved the presence of these coliforms in groundwater, surface. and drinking water samples (Omari & Manu 2012; Somaratne & Hallas 2015).

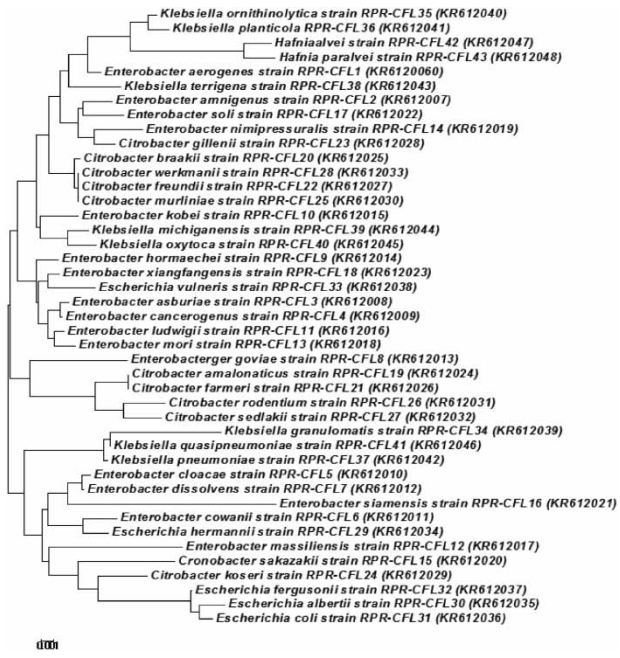


Figure 2 | Phylogenetic analysis of bacterial 16S rRNA in Ramanpadu water sample.

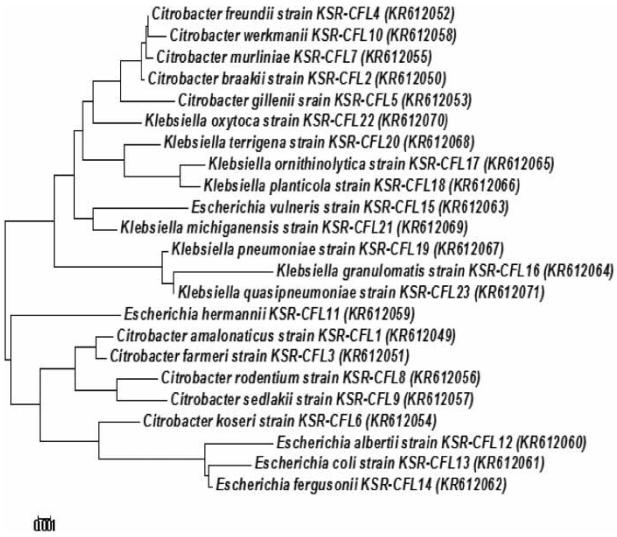


Figure 3 | Phylogenetic analysis of bacterial 16S rRNA in Koilsagar water sample.

The present study revealed the predominance of *Enterobacter*, the only representative in the order Enterobacteriales of the class Gamaproteobacteria in the phylum *Proteobacteria* (representing 10%) clones in Koilsagar rather than in Raman Pahad, indicating that the former had greater bacterial burden than Raman Pahad water.

Diarrheagenic *Escherichia coli* (DEC) are important bacteria responsible for a number of waterborne gastroenteritis outbreaks (Swerdlow *et al.* 1992), and has been detected in various ecological niches ranging from mammalian intestines to various aquatic environments, such as surface water and groundwater (Coleman *et al.* 2013). In general, an outbreak is likely to be attributed to a single *E. coli* clone as an etiological agent; however, multiple DEC strains are occasionally involved in a waterborne outbreak (McCall *et al.* 2010; Lienemann *et al.* 2011). In the present study, 12.20% and 21.42% *E. coli* were found in Raman Pahad and Koil Sagar reservoirs, respectively. Similar results were reported from environmental samples in waterborne gastroenteritis outbreaks, mainly caused by DEC, hemorrhagic colitis (Park *et al.* 2018) *E. coli* is universally used as a bacterial indicator of fecal contamination in water, the isolation of which from reservoir samples indicate that the water might have been mixed with feces (Saxena *et al.* 2015).

Citrobacter is named for its ability to utilize citrate as the sole carbon source. *Citrobacter* is reported to occur in environments such as water, sewage, soil, and food. These species can be isolated from different clinical sites, in particular, *C. freundii* is an intestinal inhabitant of humans that may sometimes produce an enterotoxin and thus become an intestinal pathogen (Frederiksen 2005). *C. amalonaticus, C. freundii*, and *C. koseri* (*C. diversus*) were reported in the feces of Australian mammals. This genus was also reported in the water of Paradi (Gordon & FitzGibbon 1999; Mukherjee *et al.* 2016). In the present study, around 25.03% and 32.14% *Citrobacter* was found in Raman Pahad and Koil Sagar reservoirs, respectively.

In spite of being recognized as naturally occuring species in the environment, the virulence properties of aquatic-borne *Klebsiella pneumoniae* which resemble clinical strains remain principally mysterious (Podschun *et al.* 2001). This bacteria causes a wide range of infections, including pneumonias, urinary tract infections, bacteremias, and liver abscesses and is the leading cause of neonatal sepsis in developing countries (Paczosa & Mecsas 2016). In the present study, *Klebsiella* was found to be around 17.86% in Raman Pahad reservoir. Oropharyngeal colonization could act as the main reservoir for nosocomial outbreaks caused by *K. pneumoniae* (Ballén *et al.* 2015).

Hafnia sp. (formerly *Enterobacter hafniea*) in the present study was found only in Raman Pahad reservoir. It resides in the gastrointestinal tract of humans and many animal species and is also found in various ecological samples. However, it is not a common human pathogen but is often associated with gastroenteritis (Janda & Abbott 2006; Aishvarya *et al.* 2017). There have been numerous reports involving diarrhea in the isolation of *H. alvei* from stool samples because the organism is part of normal fecal microbiota. However, in a case-control study of Finnish tourists returning from Morocco, the pervasiveness of *H. alvei* in those with diarrhea was notably greater than in those without diarrhea, substantiating a possible etiologic role (Ridell *et al.* 1994).

Enterobacter found in Raman Pahad reservoir were rarely found as pathogens, but these organisms are now increasingly encountered, causing nosocomial infections such as urinary tract infections and bacteremia. *E. amnigenus* has been mostly isolated from water, but some strains have been isolated from clinical specimens of the respiratory tract, wounds, and feces (Grimont & Grimont 2005).

CONCLUSION

Despite the high genetic variability of the total coliform group, this study showed that it is possible to use molecular assays to detect total coliforms in drinking water. The 16S rRNA molecular-based assay proved to be as sensitive as recommended, culture-based methods. In this study, the identification of bacterial communities in Raman Pahad and Koilasagar water samples of Mahabubnagar district, Telangana, was performed using culture-independent methods based on the 16S rRNA gene clone library. Data revealed that the Raman Pahad water has a greater diversity of coliforms as compared to the water samples from Koilsagar reservoir. The results obtained in the present study are applicable only to drinking water samples. Results could be diverse with other types of water.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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