Research Paper

Exploration of bacterial contaminants in reservoirs of Palamuru by PCR-DGGE method: household water treatment

A. Shiva Shanker, R. Kannaiah Goud and Pavan Kumar Pindi

ABSTRACT

This study is principally aimed at the exploration of the bacterial contaminants that are prevalent in the drinking water of different reservoirs in Mahabubnagar, TS, India, along with cost-effective water treatment using palatable leaves of *Mentha piperita*. Water samples from three different reservoirs of Palamuru, namely, Ramanpadu reservoir (RPR), Koilsagar reservoir (KSR) and Jurala reservoir (JUR), were collected and bacteriological and chemical parameters were assessed. Colony forming units (CFU) were determined by plate culture method using different media. The bacterial diversity concomitant with the three different reservoirs was examined and compared using a molecular approach coalescing fingerprinting by denaturing gradient gel electrophoresis (DGGE). Overall, 37 16S rDNA sequences were obtained. The sequencing dominant bands validated that the major phylogenetic groups identified by DGGE belonged to *Bacillus*, *Brevibacillus*, *Exiguobacterium*, Gammaproteobacteria, *Acinetobacter* sp. and some uncultured or unidentified bacteria. At household level, incubation of potable water with pudina leaves decreased the bacterial contamination showing its anti-bacterial effect and proving it to be an easy method to impede bacterial contamination in potable water.

Key words | bacterial contamination, bacterial diversity, CFU, community drinking water, drinking water

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ABBREVIATIONS

CFU	colony forming units
DGGE	denaturing gradient gel electrophoresis
DW	drinking water
JUR	Jurala reservoir
KSR	Koilsagar reservoir
RPR	Ramanpadu reservoir

INTRODUCTION

Human health is closely related to the quality of drinking water and access to safe, drinkable water in sufficient quantities for drinking and cooking is fundamental to ensure health and wellness (Ternes *et al.* 2002; Huerta-Fontela *et al.* 2008; Sćiban *et al.* 2009). The World Health Organization (WHO) considers that 'drinking-water' should be 'suitable for human consumption and for all usual domestic purposes including personal hygiene'. Public health problems such as waterborne and vectorborne diseases arise due to the confluence of reservoir and other water into the drinking water (Ashbolt 2004; WHO 2008; Pavan Kumar *et al.* 2013a, 2013b, 2013c). The assertion of public health implications because of bacterial prevalence in water was raised after the introduction of plate counts

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for assessing water quality by Robert Koch in 1883. In order to answer this question, we must consider historical, current, and future developments in our understanding of bacterial counts for the purposes of hygienic assessment of drinking water quality (Sharma *et al.* 2003; Feng *et al.* 2004). Drinking water is a major source of microbial pathogens in developing regions, although poor sanitation and food sources are integral to enteric pathogen exposure (Lee 2004; Zeng *et al.* 2015). Lack of safe drinking water and adequate sanitation measures leads to a number of diseases such as cholera, dysentery, Salmonellosis, and typhoid which claims millions of lives every year in the developing countries (Devi *et al.* 2008). Groundwater is the main source of drinking water in the reservoirs without any treatment (Venkata Mohan *et al.* 1996).

One of the greatest challenges of the 21st century is to provide an adequate supply of safe drinking water for household consumption to everyone. However, the quality of water resources are unevenly distributed over the Earth's surface and this is deteriorating due to anthropogenic activities, so in future countries are going to suffer from a scarcity of pure water (Pavan Kumar *et al.* 2013a, 2013b, 2013c; Bayeh *et al.* 2017). In order to protect health it is necessary to understand the quality of water supplied and consumed. For effective usage and documentation, water can be classified based on the physicochemical parameters and supplied according to various purposes such as drinking, agriculture, industrial, etc. (Chu *et al.* 2011; Pavan Kumar 2011). The biological contamination in drinking water is a major problem for public health in the developing world (Venkata Mohan & Kanniah Goud 2012). The WHO estimates that about 1.1 billion people drink unsafe water globally and a vast majority of diarrheal disease in the world (88%) is attributable to unsafe water, sanitation, and hygiene (WHO 2011).

Conventional techniques have therefore been rendered less efficient at detecting the presence of important waterborne pathogens. Hence, cultivation-independent assessment of bacterial diversity is essential – where the results are fast and at high throughput. During the last decade, the use of molecular methods has supplied the means for examining microbial diversity and detecting specific organisms without the need for cultivation. The traditional herbal medical system has been practiced globally since ancient times, and consequently, a great volume of literature is available on the antimicrobial activity of a variety of plant species (Stewart & Franklin 2008; Luo *et al.* 2013). The increased use of antibiotics has resulted in adaptation and development of resistant bacteria. In this context, different parts of the plants, herbs, and spices have been used for many years to prevent infections. These are easily available and can be used in a domestic setting for self-medication. Pudina *(Mentha piperita)* is considered as one of those medicinally important perennial herbs that belong to the family Labiate.

The aim of the present study is to investigate bacterial contaminants and compare the bacterial diversity in the drinking water of different reservoirs in Mahabubnagar along with the water treatment at household level by employing extract of *Mentha piperita* (pudina) leaves. For this purpose, the bacteriological parameters and chemical parameters were evaluated and CFU was determined by plate culture method using different media. In addition, analyses and comparison of the bacterial communities by denaturing gradient gel electrophoresis (DGGE) was performed and compared.

METHODS

Sampling and physicochemical analysis

Nine drinking water samples were collected from different drinking water sources in three different reservoirs, namely, Ramanpadu, Koilsagar, and Jurala in Palamuru, Telangana State, India in UV sterilized 1 liter water bottles. The pH of the sample was measured immediately after sampling. The samples were collected in autoclaved bottles under aseptic conditions from different sources in a manner to avoid contamination of water with other atmospheric bacteria. The sample bottles were labeled with details of the source of water, time, and date of collection. The samples were transported to the laboratory in a cool container protected from light within 6 h of sample collection for assessment of their physiochemical parameters. Physical and chemical parameters of water samples were evaluated by using the multiparameter water testing kit method (WT023, Octo Aqua Test Kit). Various physicochemical parameters, pH, turbidity, total organic carbon (TOC), conductivity, nitrate, nitrite, and ammonia were assessed (APHA 1998).

Microbial count

Total plate count can indicate the total count of bacteria in water. Therefore, total plate count is an important parameter indicating whether the drinking water has been polluted by microbes, and can be used essentially to assess the disinfection effect.

Microbial community analysis

DNA extraction and PCR amplification

The present study deals with the rapid and simple isolation of heterologous genomic DNA by manually prepared single extraction lysis mixture, including extraction buffer, lysozyme and SDS, at an optimal temperature of 55 °C. Then the DNA from the lyses of biological filters was subjected to strong acid (3 M HCl) and low base (0.1 M NaOH) treatment followed with purification using phenol-chloroform-isoamyl alcohol ethanol purification for protein and other contaminants, which gives high DNA yields. An additional advantage of this method is that 2 h of time is sufficient to isolate a large quantity of heterologous genomic DNA from biological filters. Other DNA isolation kits, namely, Qaigen Dneasy Mini Kit, Fast DNA Spin Kit, QIAMP DNA Blood Mini Kit, and conventional DNA isolation methods have also been used. However, the novel DNA isolation developed by us gave the maximum yield of DNA (Pavan Kumar *et al.* 2013a, 2013b, 2013c; Sadam *et al.* 2017). Spectrophotometric A260/A280 ratios of the final water DNA were 1.92 and 1.80. After DNA extraction, the variable V3 region of 16S rDNA was amplified by polymerase chain reaction (PCR) with primers to conserved regions of the 16S rRNA genes. The nucleotide sequences of the primers were as follows: primer 517R, 5′-ATT ACC GCG GCT GCT GG-3′; primer 341F, 5′-AGG CCT AAC ACA TGC AAG TC-3′; GC clamp was added to primer 63GC, 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAG GCC TAA CAC ATG CAA GTC-3′. The GC-rich sequence attached to the 5′-end of forward primer prevents the PCR products from complete melting during separation via DGGE (Muyzer *et al.* 1993). An automated thermal cycler (BioRad) was used for PCR amplification with the program of an initial denaturation at 96 °C for 5 min, 32 cycles of denaturation (30 sec at 93 °C), annealing (55 sec at 52.2 °C) and extension (1 min at 72 °C), and a final extension at 72 °C for 6 min. Finally, amplified PCR product was stored at 4 °C. The samples were verified in a 1% agarose gel.

DGGE screening

DGGE was performed using the DCode™ Universal Mutation Detection System (Cleaver Scientific Ltd). Samples containing approximately equal amounts of PCR amplicons (40 μL) were loaded onto 1 mm thick vertical gels containing 8% (w/v) polyacrylamide with a linear gradient of denaturants (formamide and urea). A denaturing gradient of 40–70% was applied to separate 16S rDNA fragments (where 100% denaturant is defined as 6 M urea and 40% (v/v) formamide). Gels were prepared in 1 × TAE buffer (pH 8.0, Tris, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 10 mM acetic acid, 20 mM) which was also used as the electrophoresis buffer. Electrophoresis was run at 55 °C, initially at 200 V for 20 min and then at a constant voltage of 120 V for 10 h (Venkata Mohan *et al.* 2010; Kanniah Goud & Vrenkata Mohan 2012). After electrophoresis, the gels were stained with ethidium bromide (0.6 mg/L in TAE buffer) for 10 min followed by de-staining in TAE buffer for 15 min. Images were captured using Molecular Imager G: BOX EF System (BioRad).

DNA sequence and phylogenetic analysis

The designated DGGE bands were excised with a sterilized surgical blade. The excised gels were incubated individually in 40 μL of sterile double distilled water for 24 h at 40 °C. After incubation, 10 mL of eluted DNA was used as the template for PCR performed under the conditions described as above, except that the forward primer lacked the GC clamp. A 5 mL sample of each PCR product was subjected to agarose gel electrophoresis to confirm product recovery and to estimate product concentration. The obtained amplified products were sent to Bio-serve Biotech, Hyderabad for sequence analysis. All the 16S rDNA sequences were aligned with those of the reference microorganisms in the same region of the closest relative strains available in the online GenBank database by using the BLASTN facility (http://www.ncbi.nlm.nih.gov/BLAST/). Molecular

Evolutionary Genetic Analysis, MEGA version 6.0 was used to align the sequences with the closest matches found in the online GenBank database with the CLUSTALW and construction of neighbor-joining phylogenetic tree. A bootstrap analysis with 600 replicates was carried out to check the robustness of microbial consortia present in the phylogenetic tree.

Nucleotide sequence accession numbers

Sequences were submitted to the Nucleotide Sequence database to the online GenBank public database under the accession numbers LT592288 to LT592327.

Plant materials and extraction

Indigenously grown pudina plants were collected from local village markets of Mahabubnagar. The plant material was thoroughly washed with clean water to remove soil and other dirt. Then, the leaves were separated and air dried for complete drying. The dried plant material was powdered using a hand blender. The powder was extracted with methanol according to the maceration method and the extract was filtered by Whatman no.1 filter paper. The filtrate was concentrated in a rotary evaporator at 40 °C. The concentrated extract was oven dried at 40 °C for 3 days and freeze dried for 48 h. The freeze dried extracts were stored at −20 °C until use (Figure S1, available with the online version of this paper).

Microbial cultures and growth conditions

Bacterial pathogens such as *Staphylococcus aureus, Proteus vulgaris, Salmonella typhi* A and B, *Escherichia coli, Citrobacter, Enterobacter, Proteus aeruginosa*, and *Klebsiella* pneumonia obtained from IMTECH, Chandigarh were used as test organisms. Cultures of bacteria were grown for 10 h in nutrient broth at 37 °C and were maintained on nutrient agar slants at 4 °C.

Antimicrobial activity assay by disk diffusion method

The dried plant extract was dissolved in methanol to a final concentration of 1 mg/mL. The leaf extracts in methanol were filter sterilized using membrane filter (pore size 0.47 μm). The bacterial strains were grown on nutrient agar (NA) then later in Mueller Hilton Broth (MHB). The final bacterial concentration was adjusted to 0.5 McFarland standard turbidity. This bacterial culture was used for plating onto Muller Hinton Agar (MHA) plates. Sterile Whatman filter paper (no.1) disks of 6 mm in diameter were impregnated with 10 μL of crude extract at 1 mg/mL prepared using methanol. The disks were evaporated at 37 °C for 24 h. The plates were air-dried under a sterile hood and the impregnated disks were placed at equidistant points on top of the agar medium. A disk impregnated with methanol was used as negative control. Antimicrobial activity was evaluated by measuring the diameter zone of inhibition around the disk.

RESULTS AND DISCUSSION

Water samples from different reservoirs of Palamuru, namely, Ramanpadu, Koilsagar, and Jurala during different seasons were collected and the CFU was compared on different bacteriological media. For each sample, bacteriological parameters and chemical parameters were evaluated and CFU was determined by plate culture method using different media. The bacterial diversity associated with the three different reservoirs was investigated and compared using a molecular approach combining fingerprinting by DGGE.

Physicochemical analysis and CFU

Three water samples were collected from different reservoirs of Palamuru, namely, Ramanpadu, Koilsagar, and Jurala during different seasons. Physical parameters of water samples showed varied turbidity, pH, hardness and different chloride, fluoride, nitrate, and iron concentrations (Table S1, available with the online version of this paper); CFU is a measure of viable bacterial cells. For convenience, the results are given as CFU/mL (colony-forming units per milliliter) for liquids. The mean total bacterial counts of each water sample ranged from 9.5×10^7 CFU/mL, $8.0 \times$ 105 CFU/mL, and 6.5×105 CFU/mL for Ramanpadu, Koilsagar, and Jurala, respectively (Figure 1). Although there were differences in the average total bacterial counts of

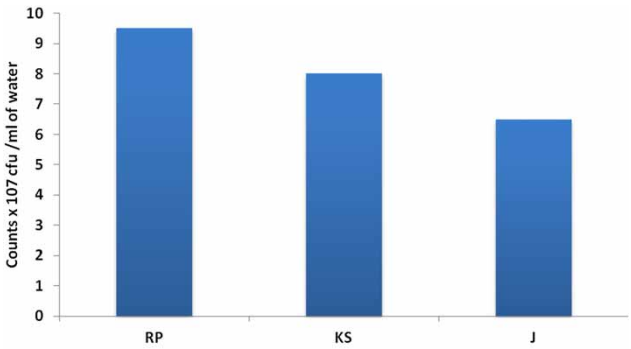

Figure 1 | Average total bacterial count (TBC) of the sampling locations. RP: Ramanpadu, KS: Koilsagar and J: Jurala.

different sampling locations, these differences were not statistically significant. However, the highest counts were observed in Ramanpadu, then Koilsagar and the lowest count was observed in Jurala.

DGGE community analysis

The microbial diversity of the water samples from different reservoirs of Palamuru, namely, Ramanpadu, Koilsagar, and Jurala was analyzed by PCR-DGGE techniques (Figure 2). The bands obtained after gel running were elucidated and sent for sequencing. Sequences were submitted to the Nucleotide Sequence database of the GenBank public database. DGGE analysis at V3 region of 16S rDNA demonstrated a clear banding pattern of the microbial community structure as well as denoting the contaminant species richness in three water samples (Figure 3).

The phylogenetic distributions established with a bootstrap neighbor-joining method are depicted in Figure 4. Each band on the DGGE profile corresponded to a gene

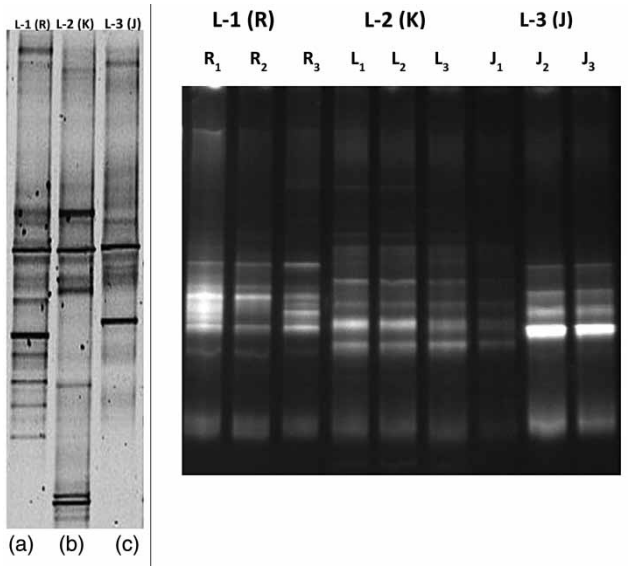

Figure 2 | Variation of DGGE profiles with three different reservoirs: (a) bands in lane 1, RPR; (b) bands in lane 2, KSR; (c) bands in lane 3, JUR (Imager G:BOX EF System; Syngene).

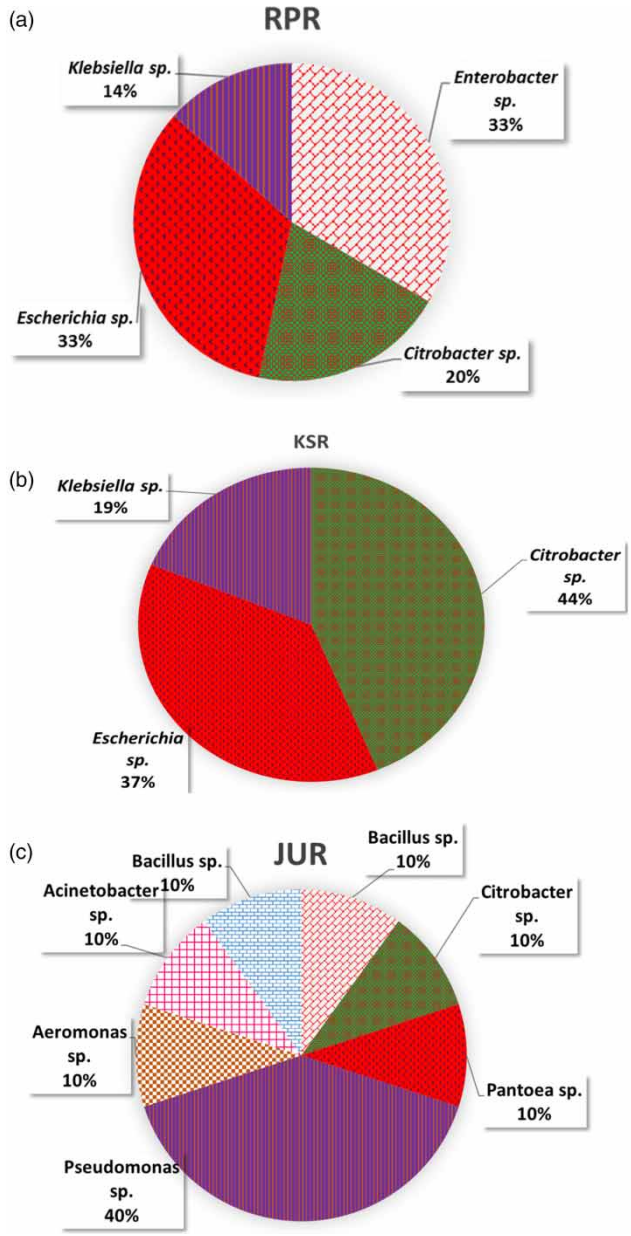

Figure 3 | Three different reservoirs in Mahabubnagar, T.S., India. Phylogenetic community comparison with the function of experimental variations studied. (a) RPR: Ramanpadu; (b) KSR: Koilsagar; (c) JUR: Jurala.

Figure 4 | Neighbor-joining tree constructed using Mega 4.0 showing phylogenetic relationships of 16S rDNA sequences from closely related sequences from GenBank. Three different reservoirs in Mahabubnagar, T.S., India. (a) RPR: Ramanpadu; (b) KSR: Koilsagar; (c) JUR: Jurala.

Table 1 | Three main reservoirs of phylogenetic sequence affiliation and similarity to the closest relative of amplified 16 rDNA sequence excised from DGGE

DGGE band number	Closest match	Different bacterial sp. with accession no.			Similarity (%)	Phylogenetic affiliation (Class)
		RPR	KSR	JUR		
DW – 1	*Enterobacter* sp.	LT592288; LT592311; LT592312; LT592320; LT592321			93	Gammaproteobacteria
DW – 2	*Citrobacter* sp.	LT592289; LT592313; LT592322	LT592293; LT592296; LT592297; LT592314; LT592304; LT592305; LT592306	LT592315	99	
DW – 3	*Escherichia* sp.	LT592290; LT592316; LT592318; LT592323; LT592324	LT592291; LT592298; LT592299; LT592300; LT592307; LT592317		98	
DW – 4	*Klebsiella* sp.	LT592319; LT592302	LT592292; LT592301; LT592303		96	
DW – 5	*Pantoea* sp.			LT592294	100	
DW – 6	*Pseudomonas* sp.			LT592295; LT592327; LT592310; LT592309	99	
DW – 7	*Aeromonas* sp.			LT592308	96	
DW – 8	*Acinetobacter* sp.			LT592325	98	
DW – 9	*Bacillus* sp.			LT592326	98	Bacilli

fragment of unique 16S rDNA sequences and accordingly represented a specific species in the microbial community. Dominant operational taxonomic units (OTUs) observed in the three drinking water samples could be divided into two groups, Proteobacteria and Firmicutes (Table 1). Major bands were phylogenetically related to class Gammaproteobacteria, a class of several medically, ecologically, and scientifically important groups of bacteria. An exceeding number of important pathogens belong to this class, e.g., *Salmonella* spp. (enteritis and typhoid fever), *Yersinia pestis* (plague), *Vibrio cholerae* (cholera), *Pseudomonas aeruginosa* (lung infections in hospitalized or cystic fibrosis patients), and *Escherichia coli* (food poisoning). The majority of the DGGE bands from Koilsagar reservoir was found to be associated with Clostridia and Bacilli, each with four OTUs out of thirteen. Koilsagar reservoir dominates with 17 OTUs, representing three genera. DW-2 was found to have 99% similarity with the *Citrobacter* sp. with seven OTUs and followed by DW-3 which was found to have 98% similarity with the *Escherichia* sp. with five OTUs. Out of the three DGGE bands in Koilsagar, the third one exhibited 96% similarity with *Klebsiella* sp. (three OTUs). The second most predominant bacterial contaminants were found in Ramanpadu reservoir with 15 OTUs.

DW-1 with five OTUs exhibited 93% similarity with *Enterobacter* sp. DW-2 was found to have 99% similarity with the *Citrobacter* sp., with three OTUs. DW-3 exhibited 98% similarity with *Escherichia* sp. OTUs and DW-4 was found to be similar with *Klebsiella* sp., with two OTUs. The least bacterial contaminants were found with Jurala reservoir exhibiting the least total number with nine OTUs with six different OTUs. DW-2 exhibited 93% similarity with *Citrobacter* sp., DW-5 100% similarity with *Pantoea* sp., DW-6 99% similarity with *Pseudomonas* sp., DW-7 96% similarity with *Aeromonas* sp., DW-8 98% similarity with *Acinetobacter* sp. and DW-9 96% similarity with the *Bacillus* sp. and were present only in Jurala reservoir; whereas *Citrobacter* sp. was present in all three reservoirs,

and *Enterobacter* sp. was present only in RPR. The RPR, KSR, and JUR are all contaminated with *Citrobacter* whereas RPR and KSR are both dominated by *Escherichia* and *Klebsiella*. RPR dominates with 17 species, followed by KSR with 15 and JUR with eight species, respectively. On the whole, bacterial diversity was found to be more diversified with different types of bacterial contaminants in Ramanpadu water followed by Koilsagar and Jurala. CFUs were also found to be highest in Ramanpadu followed by Koilsagar and Jurala, indicating high levels of bacterial contaminants rendering water unsafe for human consumption.

Antimicrobial activity assay

The evaluation of antimicrobial potency by the disk diffusion method indicated that all the bacterial strains tested have shown growth inhibition toward the plant extract with differing sensitivity. Among the bacterial pathogens, *Staphylococcus aureus, Proteus vulgaris, Salmonella typhi* A and B, *E. coli, Citrobacter, Enterobacter, Proteus aeruginosa* and *Klebsiella pneumonia* have displayed a higher inhibition zone demonstrating the efficacy of the pudina's leaves in exhibiting anti-bacterial properties (Figure 5).

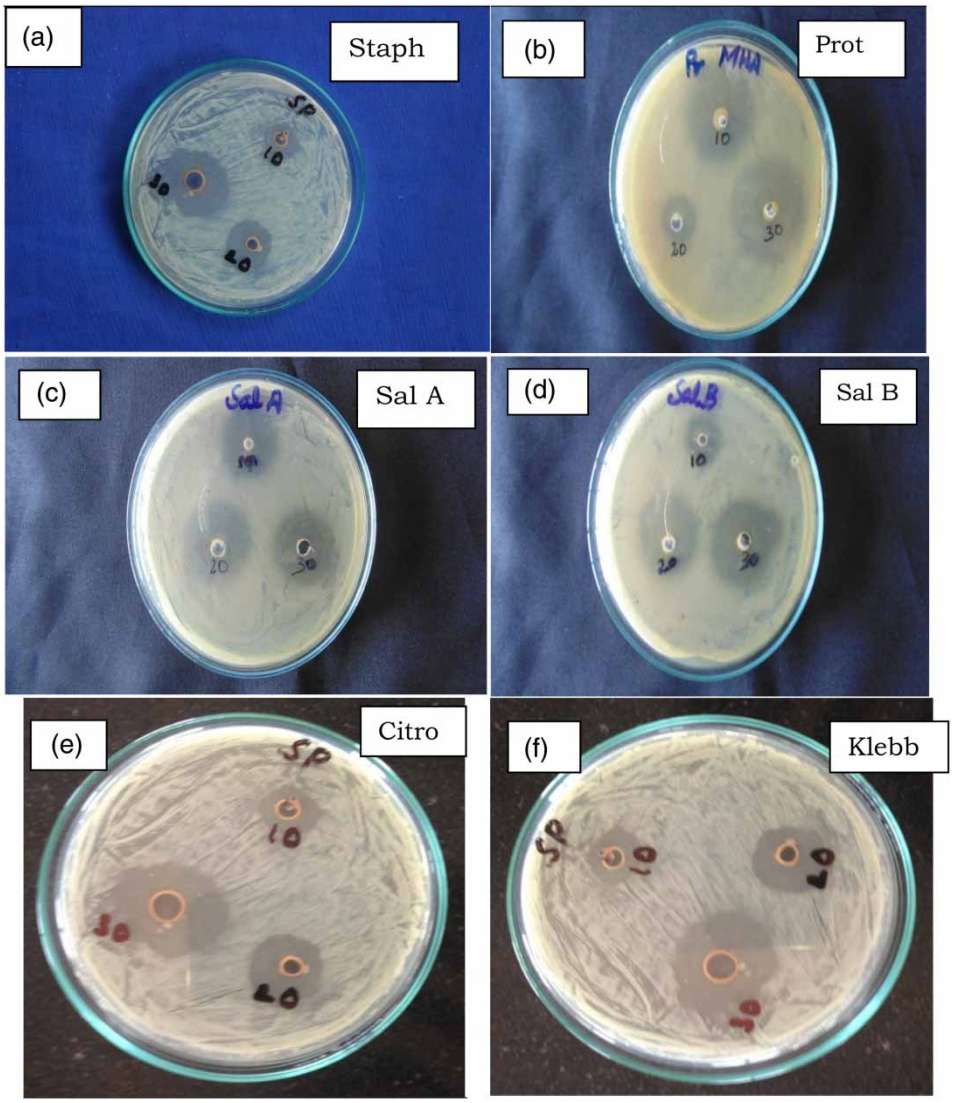

Figure 5 | Anti-bacterial effect of *Mentha piperita* on different water bacterial contaminants. (a) Staph: *Staphylococcus aureus*; (b) Prot: *Proteus vulgaris*; (c) and (d) Sal: *Salmonella typhi* A&B; (e) Citro: *Citrobacter*; and (f) Kleb: *Klebsiella pneumonia*.

SPECIAL DISCUSSION

The study of bacterial contamination levels in potable water bodies is importment and essential to understand.

Conventional culture-based microbiological water quality monitoring techniques take a long time (several days) and usually a small volume of water is sampled (typically 1 mL). This results in inadequate detection limits (only 1% of the water pathogens can be identified with regards to drinking water safety), rendering the conventional techniques more difficulting in detecting the presence of important waterborne pathogens.

Therefore, cultivation-independent assessment of bacterial diversity is essential, in which the results are faster and at high throughput (Ng *et al.* 2015). The PCR–DGGE-based approach applied here has been shown to be effective in establishing new data concerning the microbial contamination of these drinking water communities. Phylogenetic analysis revealed that the structure of bacterial contaminates communities in both Ramanpadu and Koilsagar reservoirs were very similar. Bacterial contaminant communities were composed of organisms affiliated with Proteobacteria and subdivision of Gammaproteobacteria. Sequences belonging to this group have previously been retrieved from other wastewater environments. Many of them are pathogen-related with organisms involved in pollutant degradation, which suggests the importance of such communities for wastewater treatment (Petrovic *et al.* 2013). Most of the sequences retrieved showed the greatest similarity with uncultured bacteria, demonstrating once again the importance of culture-independent molecular methods for the study of wastewater communities (Hu *et al.* 2012).

As our knowledge of clinical microbiology increases and epidemiological surveillance improves, the range of microorganisms that have been shown to cause waterborne outbreaks have grown. However, the number of attributable 'sporadic' infections to water is unknown. Many of the pathogens associated with water are also transmitted by food, and thus it is difficult to determine the source of most sporadic infections. The number of different types of bacteria that have the potential to cause disease in human beings and that have been isolated from water is large, yet the incidence of infection in human beings is often extremely low, even in areas where the water distribution system is continually colonized.

However, a wide variety of 'opportunistic pathogens,' such as *Aeromonas*, *Pseudomonas* and some species of *Mycobacterium*, are commonly found. The significance of their presence in water supply in the etiology of human disease, however, is not well defined. Precautions should be taken not to contaminate the water bodies by human activities (Li *et al.* 2016). Improperly maintained treatment devices can also be a source of contamination. Home water filters and other water treatment devices should be changed and maintained in accordance with manufacturers' recommendations. Frequent monitoring of the water supplying reservoirs and other domains should be made compulsory. Bacteriological and physico-chemical parameters should be in the permissible limits approved by the WHO. Furthermore, steps should be taken by the government to see that the potable water is safe before supplying it to the public. People should also be aware of water contamination and sanitation and should take the necessary precautions to avoid contamination at household level by boiling, using edible antimicrobial leaf extracts (mint leaves, *Pinpinella thirupathensis*, etc.). The work presented here has provided these insights.

CONCLUSIONS

The results of bacterial contaminants and composition in the drinking water of different reservoirs in Mahabubnagar data revealed that the Ramanpadu water has more diversity of coliforms and bacterial contaminants compared to the water samples of Koilsagar and Jurala reservoirs. This is also highly contaminated with CFU values which do not meet the WHO/UNICEF standards. WHO/UNICEF concludes that 38% lack access to even rudimentary levels of water, 19% lack sanitation, and 35% do not have water. Also, the diversity of coliforms and non-coliforms obtained by the uncultivable methods like DGGE was more easy and rapid when compared to the cloning strategies. Since there is a vast amount of information held within the genomes of cultivable and non-cultivable microorganisms, cultivation-independent assessment of bacterial diversity is essential for throughput evaluation. There is a high need

for water monitoring programs in these reservoirs from the viewpoint of public health. In this context, different parts of plants, herbs, and spices have been used for many years for prevention of infections. These are easily available and can be used in a domestic setting for self-medication.

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