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EVALUATION OF ENTERIC BACTERIA AND ITS ASSOCIATION IN BIOFILM FORMATION IN POTABLE WATER SAMPLES

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ABSTRACT

Drinking water quality is a dynamic distress to mankind as it is directly allied with human welfare and in this sense, evaluation of enteric bacterial contamination to potable water samples was executed. Seasonal sampling was designed in the study and a total of 90 water samples were collected from three different sources of potable water of Mysuru city. The Hi-Crome coliform chromogenic agar was used as a selective, presumptive medium to isolate bacteria. Water analysis revealed the presence of H_2S producing bacteria, total coliforms count bared 6 CFU/100 ml to too numerous to count (TNTC) CFU/100 ml, respectively. Pure cultures of bacteria were characterized by biochemical tests and molecular documentation. Thus, obtained results were statistically analyzed. Sequences encompassed in this paper are deposited in the NCBI database. Screening for detection of biofilm formation by the bacterial isolates was verified and only one isolate was successfully recovered from harvested rainwater samples. The analysis exposed the water quality was well above the permissible limits of WHO for the first year and an improvement of quality in the second year. The results clearly revealed the poor quality of potable water samples. The detection of H_2S producing bacteria, a higher range of total coliforms and biofilm forming bacterial pathogens in potable water sources is a potential indicator of health hazard to person in contact with water and thus pose a risk to the community residents of the city.

Keywords: Potable water, Membrane filtration, Fecal contamination, Enteric bacteria, Biofilm bacteria

1. INTRODUCTION

One of the things which makes this earth a unique planet in this universe is continuous availability of water, a vital prerequisite for the existence of life. All the ancient citizens known to history and archaeology flourished on the banks of rivers as they were consented with the importance of water, ever since man appeared on the surface of the earth. Water is also the essential prerequisite of agriculture, the source of food desirable for the survival of life. Thus, life on earth is entirely and exclusively dependent on water [1]. Even though water covers more than 70% of the earth; only 1% of the earth's water is available as a source of drinking and maximum part of it is in polluted form [2]. Yet our society continues to pollute it. The discharge of wastes from municipal sewers is one of the most important water quality issues world-wide. It is of particular significance to sources of drinking-water. Municipal sewage contains human feces and water contaminated with these effluents may contain pathogenic organisms that may be hazardous to human health if used as drinking water or in food preparation [3].

Groundwater a gift of Mother Nature encompasses about 210 billion m^3 including recharge through infiltration, discharge and evaporation. Today human activities are constantly adding industrial, domestic and agricultural waste to groundwater reservoirs at an alarming rate. Groundwater contamination is usually irreversible that is, once contaminated it is difficult to restore the original water quality of the aquifer [4]. The lack of quality monitoring in groundwater and governing of regulations required in drilling of wells make the public consuming water that is devoid of proper treatment. It is of potential public health concern to consume water without sufficient quality control, as it can often be a vehicle in spread of disease [5].

Universally water related diseases like cholera, typhoid, amoebic and bacillary dysentery and other diarrheal diseases are caused by drinking water fouled with human or animal excretions that comprise pathogenic microorganisms. An estimated 1.8 million deaths in 2010

has resulted due to gastroenteritis (WHO), 88% due to unsafe water and poor sanitation. Current WHO bacteriological guidelines 4 for drinking-water indorsed zero fecal coliforms per 100 ml of water. The majorities of the population in developing countries are inadequately supplied with potable water and are thus bound to use water from sources like shallow wells and bore holes that have a high potential of contamination, offering the unsafe water for domestic and drinking purposes [6]. Ever increasing demand for water could be met by harvesting rainwater as it helps in reduce of run-off which is clogging storm drains, reduce flood hazards, augment groundwater storage and control the decline in water level, reduce soil erosion and groundwater quality will be improved. Thus, rainwater harvesting could be considered as an ideal solution for water problem, where there is insufficient groundwater supply or where surface water resources are either scanty or not available. Rainwater is soft in nature, bacteriologically pure and free from organic matter [7].

Although universal access to safe and piped drinking water is an important long-term solution, is quite costly and exigent to implement in developing countries in the short time [8, 9]. Microorganisms have the ability to adhere to the solid surfaces and form biofilm in aquatic environment [10]. Generally, most water distribution systems are characterised by the presence of biofilm, regardless of purity, the brand of pipe material used for distribution or the presence of a disinfectant [11].

Biofilm are the bacterial communities embedded in a polysaccharide matrix, which gives them the chance to resist destruction by antibodies, environmental stress, biocides and detergents. Bacterial regrowth in the distribution system may result from the detachment of biofilm bacteria, which increases the risk of infection in humans when water is consumed [12]. Different materials such as cast iron, galvanised steel, stainless steel, copper and polyethylene have been used to manufacture water distribution pipes and these materials favour biofilm formation in the water distribution systems. The development of biofilm in copper pipes facilitates cuprosolvency which increases the release of copper and also corrosion of copper into the distribution system [13,14]. Adverse health effects in humans, especially in children is known to occur due to the corrosion of plumbing materials made of lead and this favours lead contamination in tap water [15]. Therefore, biofilm formation is of major concern for most municipal supply agencies and communities as it results in the deterioration of the quality of drinking water. Considering these aspects, naturally occurring biofilm in contact with drinking water were identified and described as microbial reservoirs for further contamination [16]. The World Health Organization (WHO) defines safe drinking water as, "water that does not represent any significant risk to health over a lifetime of consumption, including different sensitivities that may occur between life stages" [17]. Household water treatment practice may play a vital role in protecting public health where existing water sources, including those delivered via a piped network become contaminated during distribution or storage [18].

The rapid expansion of Mysuru city has led the resident to be dependent on groundwater to reserve them with potable water. Therefore, one of the most important prerequisites in improving the health of the people living in developing countries is the provision of safe and clean water. Thus, the study was intended to evaluate the enteric bacterial eminence in potable water samples of different sources in the study area. The study highlights the hygiene, health and surrounding environmental sanitation. The work draws together the evidence from all studies of potable water contamination at the point of use and identifies how water contamination varies between different studies settings. The present investigation is an attempt to examine the water quality of regularly used potable water sources within Mysuru city.

2. MATERIAL AND METHODS

2.1. Study area

Mysuru is one of the largest city in Karnataka, a southern Indian state, of India. Mysuru is located at 12.30°N 74.65°E and has an average altitude of 770 m (2,526 ft). The city's average rainfall is 804.2 mm (31.7 in).

2.2. Collection of water samples

Evaluation of enteric bacteria was executed to the water samples intended for human consumption. Water samples were collected for a period of two years from March 2017 to February 2019, at three different sources of potable water such as, drinking water (DW) from public refreshment centers, groundwater (GW) and harvested rainwater (HRW) from five zones within Mysuru city. Water samples were collected aseptically in pre-sterilized bottles, labelled and transported immediately to the laboratory and analysed. All the

chemicals required for laboratory work were procured from Hi-media laboratories Pvt. Ltd., Mumbai, India.

2.3. Bacteriological water analysis

Screening of enteric bacteria from potable water samples was executed by isolation, enumeration and identification of bacteria according to the standard methods prescribed [19, 20]. Primarily, isolation and identification of bacterial cultures were performed based on H_2S producing bacteria, cultural characteristics and Gram's staining technique [21], further confirmation was followed by biochemical tests and molecular documentation.

2.4. Isolation, purification and characterization of enteric bacterial strains

Isolation and identification of enteric bacterial species from potable water samples was executed primarily by presence/absence test to Hydrogen sulphide producing (H_2S) bacteria using H_2S bottle (Hi-selective H_2S Medium Kit, K022- 1kit) powder form Water testing kit. Predominantly, field test to H_2S producing bacteria was made on spot at sampling place [22]. Potable water samples were collected in a pre-sterilized borosil glass bottle (500 ml) for bacteriological analysis. Water samples were aseptically collected and inoculated into H_2S bottle upto the mark on spot and transported to the laboratory in an icepack bag. Then the H_2S bottles were incubated at 37°C for 18-24 h in the laboratory incubator. After the incubation period, H_2S bottles were observed for a change in colour of the medium with turbidity.If the medium shows turbidity with bluish green / bluish purple / black colour, it indicates positive for contamination by H_2S producing bacteria in inoculated water sample.

Table 1: Appearance of HiselectiveH_2S medium kit (powder form) K022- 1kit, in response to water samples

Colour of the H_2S bottle inoculated with water sample with turbidity	Identification of bacteria
Clear bluish green	Control-clear
Bluish purple	*Klebsiella* / *Enterobacter*sp.
Bluish green	*E. coli*/*Streptococcus*/*Shigella* sp.
Black	*Salmonella* sp. /*Citrobacter* sp.

Note: Control vial appear clear bluish green against intense light. Bacterial growth is indicated by turbidity and colour change as indicated in the table 1

2.5. Standard analysis of potable water samples

Standard analysis of drinking water samples was performed by membrane filtration (MF) technique using 0.22µm pore size filter paper. After filtration, filter paper was placed aseptically over a sterile, solidified Hi-Crome coliform chromogenic agar plate and incubated at 44.5±2°C for 24-48 h. After incubation period, colony forming units (CFU/100 ml) from the plate were enumerated and total coliforms count was recorded. Isolation and enumeration of total bacteria were done in triplicates, its mean was taken and statistically analysed (Table 2). All potable water samples were inoculated undiluted over selective media [23]. Single colony was picked from coliform chromogenic agar plate and streaked on nutrient agar slants to maintain pure cultures for further analysis.

2.6. Molecular identification of bacterial isolates

Culture methods for the detection of coliforms have limitations such as long incubation period, interactions with other microorganisms, lack of accuracy and basic sensitivity and poor identification of VBNC bacteria. As an accurate and rapid method for the detection of coliforms, molecular methods have been proposed [24]. Bacterial DNA was isolated by Cetyltrimethyl ammonium bromide method [25]. Then the isolated bacterial DNA was further sequenced. Sequencing was done at Biokart India Pvt. Ltd. Ref No. BKSANG3013. The sequences obtained were submitted to National Centre for Biotechnology Information (NCBI) database.The Genbank accession numbers are listed in table 3.

Further, comparative analysis of biofilm forming bacteria from potable water samples was preceded.

A prospective study was undertaken to evaluate the water quality and bacteria involved in biofilm production in potable water samples. Potable water samples were collected and assessed for isolation and enumeration of total coliforms by Membrane filtration technique [19]. Through literature review and present investigation, the pure cultures were selected and assessed for biofilm forming potential of bacteria in water.

2.7. Identification of biofilm forming bacteria in potable water samples

Screening aimed at detection of biofilm formation by the bacterial isolates was verified by cultural methods namely, a qualitative Tube Method [26] to test bacterial potential in biofilm formation. Similarly, the biofilm

forming potential of test bacteria was detected according to Congo red agar method [27]. In a qualitative Tube Method, the test bacteria was inoculated into test tubes comprising trypticase soy broth (10 ml) mixed with 1% glucose and tubes were incubated at 37°C for 24 h. Then the content in tubes was evacuated and at pH 7.3 tubes were washed with phosphate buffer saline and dehydrated. Next, crystal violet (0.1%) stain was applied to tubes with deionized water excess stain was removed and dried by inverting tubes. The biofilm formation would be measured as positive when the test tube's bottom and walls were lined with a visible film. In Congo red agar method, the medium was made using brain heart infusion broth (37g/l), sucrose (50g/l), agar No. 1 (10g/l) and Congo red indicator (8g/l). At first, the Congo red stain solution was prepared and sterilized for 15 min at 121°C. Later, at 55°C brain heart infusion broth and sucrose was sterilized and mixed with stain. Finally, the test bacteria were inoculated on Congo red agar plates and aerobically incubated at 37°C for 24 h. Colonies appearing as dry crystal-like consistency with black colour indicate biofilm production.

3. RESULTS AND DISCUSSION

The potable water samples collected from Mysuru city were analysed for bacteriological quality of potable water sources that was regularly used in the city (Table 2). Detection of H_2S producing bacteria as an indicator of fecal contamination is considered as more efficient compared to coliform test in water [28]. Contamination of potable water samples by H_2S producing bacterial pathogens to all three types of potable water samples was identified. A majority of potable water samples were contaminated with *Klebsiella / Enterobacter*sp. (23), *E. coli / Streptococcus / Shigella* sp. (19), *Pseudomonas* sp. (18), *Vibrio* sp. (13) and *Salmonella* sp. / *Citrobacter* sp. (4) in different seasons. The investigation was successful in detecting H_2S bacteria (85.5%) in potable water samples. Similarly, the study showed more positive H_2S test (78%) compared to coliform and fecal coliform tests (59%), mainly with hand pump water samples and pipe supplies [29]. Presence of fecal pollution and H_2S producing bacteria in water has a strong correlation which indicates the presence of H_2S producing bacteria consistently in feces. Thus, fecal pollution of water can be recognized by detecting H_2S forming bacteria in water samples [30].

Table 2: Summary of total coliforms isolated and enumerated from potable water samples from five zones of Mysuru city, March 2017 to February 2019

Season	Water	Zones										Total	
		East		West		South		North		Central			
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D
Pre Monsoon	DW	31.50	21.38	16.17	17.71	79.17	15.55	16.33	17.91	59.83	56.42	40.60	37.76
	Ground	17.17	6.15	16.00	7.40	199.17	127.17	30.00	21.55	19.67	12.09	56.40	90.59
	Harvest	18.33	7.74	2.50	1.87	10.17	1.60	0.00	0.00	37.67	5.50	13.73	14.40
	Total	22.33	14.42	11.56	12.36	96.17	106.23	15.44	19.75	39.06	35.69	36.91[q]	59.33
Monsoon	DW	23.67	1.63	7.67	8.43	10.17	1.33	18.83	3.97	48.17	43.64	21.70	23.66
	Ground	18.00	5.76	16.67	6.25	159.50	41.83	40.67	32.51	22.67	4.80	51.50	59.94
	Harvest	25.33	7.74	3.67	2.42	12.17	5.04	0.00	0.00	73.17	11.55	22.87	27.78
	Total	22.33	6.21	9.33	8.09	60.61	75.50	19.83	24.65	48.00	32.50	32.02[q]	42.39
Post Monsoon	DW	15.83	1.83	0.00	0.00	12.50	1.22	11.67	0.52	12.50	1.22	10.50	5.64
	Ground	6.33	6.98	5.83	6.46	83.50	19.91	12.83	10.44	18.00	11.37	25.30	31.97
	Harvest	17.00	5.55	1.50	1.38	6.83	2.48	0.00	0.00	27.33	4.97	10.53	10.99
	Total	13.06	6.97	2.44	4.40	34.28	37.51	8.17	8.23	19.28	9.24	15.44[p]	20.78
Total	DW	23.67	13.40	7.94	12.62	33.94	34.00	15.61	10.41	40.17	43.89	24.27	28.52
	Ground	13.83	8.07	12.83	8.12	147.39	88.46	27.83	24.87	20.11	9.58	44.40	66.08
	Harvest	20.22	7.64	2.56	2.04	9.72	3.89	0.00	0.00	46.06	21.53	15.71	19.64
	Total	19.24[a]	10.68	7.78[a]	9.57	63.69[c]	80.96	14.48[a]	19.12	35.44[b]	30.36	28.13	44.57

Note: Mean values with different superscripts are significantly different from each other as indicated by Scheffe's post hoc test (alpha=.05). DW=Drinking water, GW=Ground water and HRW=Harvested Rain Water

Isolation and enumeration of total coliforms revealed a highest mean total coliforms (Too numerous to count-TNTC) contamination in groundwater samples from south zone and this may be due to the close proximity of sewage water treatment plant and ground water source. Very lowest mean total coliforms of 7.78 CFU/100 ml and 14.48 CFU/100 ml count was observed in harvested rainwater samples from west zone and north zone of the city respectively. Thus harvested rainwater samples presented a better quality of potable water, may be due to vigilant maintenance of harvested rainwater structures at residential level. A total of 184 CFU/100 ml in sample 1, 168 CFU/100 ml in sample 2, in sample 3 there was 172 CFU/100 ml and 187 CFU/100 ml in sample 4 was recorded by membrane filtration technique from the water samples collected from Shivanath River [31]. WHO indorses 10 MPN 100 ml^{-1} of coliforms and nonefecal coliforms in drinking water [32].

Molecular techniques are accurate, swift and sensitive methods for the study of specific pathogenic bacteria. These tools can be used for an exact analysis of the drinking water performance at eliminating pathogens in drinking water and water treatment plant [33]. Based on alignment of sequence and phylogenetic analysis the bacterial isolate has matched to *E. coli* and *K. pneumoniae*. Further, the sequences of National Centre for Biotechnology Information (NCBI) database were used and compared with sequences of bacterial isolates. The Genbank accession numbers of bacterial cultures are MT230530 and MT192344 for *E. coli* and *K. pneumonia*, respectively.

Table 3: Summary of sequences analyzed to bacterial cultures isolated from potable water samples

Sample description	Sample prefix	NCBI accession number
Ground water	GW	MT230530
Harvested rainwater	HRW	MT192344

NCBI-BLAST search results presented a highest sequence similarity with *Escherichia coli* and *Klebsiella pneumoniae* and the accession number were as MT230530 and MT192344, respectively.

Analysis of biofilm forming bacterial network was undertaken to potable water samples. Bacterial isolates obtained from the work was investigated to assess the biofilm forming potential in water. The H_2S bottle inoculated with groundwater sample showed bluish green colour with turbidity and harvested rainwater sample showed green colour and turbidity specifying the presence of *Escherichia coli* and *Klebsiella / Enterobacter* sp. contamination, respectively (Himedia, H_2S bottle-K022). Thus, the presence of H_2S producing pathogenic bacteria was confirmed as *E. coli* and *K. pneumoniae* by molecular method. Further, biofilm forming potential of bacteria were analyzed by Tube method and Congo red agar method. Culture tubes with white slime layer attached to the tube (Fig.1) walls were observed where one tube was with strong slimy layer and the other tube had a weak or narrow slimy layer formation. Here *Klebsiella pneumoniae* from harvested rainwater sample was the only tube which showed slimy layer indicating positive to biofilm production and *Escherichia coli* sp. was a weak or negative to biofilm production.

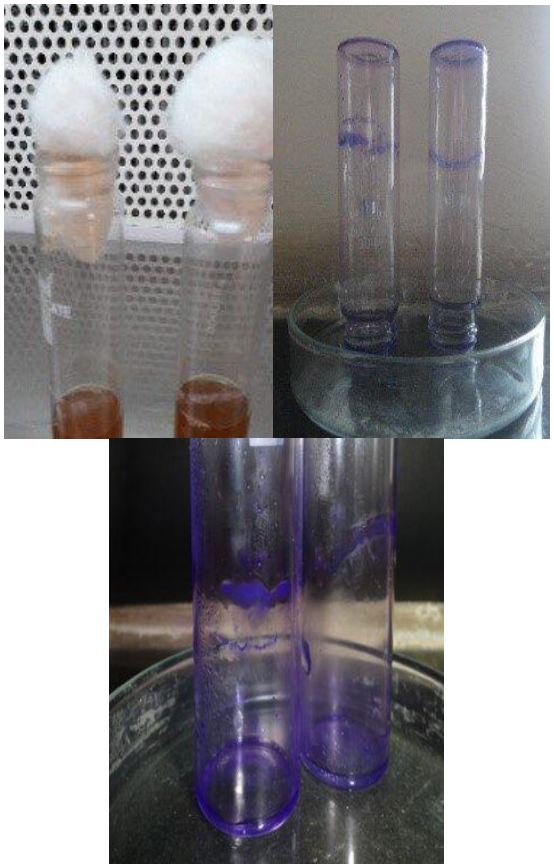

Fig. 1: Screening for detection of biofilm formation study by Tube method

This method confirmed that *Klebsiella* isolate from harvested rainwater sample formed a reasonable and steady biofilm at 44.5°C by tube method; whereas, *E. coli* was weak or narrow to biofilm formation in groundwater

samples. In Congo red agar plate, a single, small black colony and a stretch of black consistent crystalline colony is seen which confirms the presence of biofilm bacteria in harvested rainwater sample (Fig. 2). Similarly, *Klebsiella* isolate formed a moderate consistent biofilm at 44.5°C than 35°C as measured by CRA and Tube method [34]. On Congo red agar media plate, development of black crystalline colonies were measured to be positive isolate, whereas colourless colonies as negative to biofilm production [27]. Diverse bacterial species along with *Pseudomonas aeruginosa* association in biofilm have been noticed within water distribution systems in countries which have added progressive water-treatment facilities [35, 36].

Positive culture tube with white slimy layer attached to the tube walls; negative tube shows very thin or no slimy layer (Fig. 1), Biofilm positive-Black consistent crystalline colonies, and negative colourless colonies (Fig. 2).

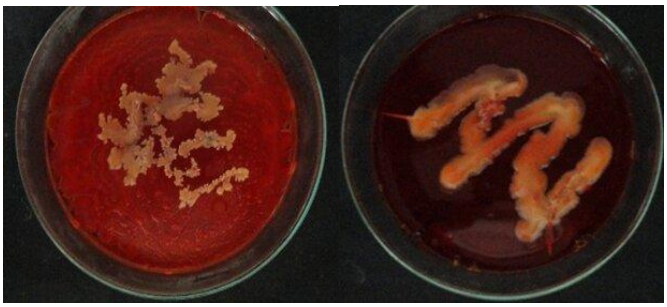

Fig. 2: Biofilm growth study by congo red agar method

4. CONCLUSION

Water is known to be one of the significant resources for all organisms to all utilities on the earth, involving plants, microbes and animals. Thus, the present study is prompting to study the quality of water and its availability. In this planet water is the most precious and indispensable natural resources. The inherent quality of potable water in Mysuru city of Karnataka, India is quite low and a proper socioeconomic policy and environment to improve and maintain water quality is lacking. Anthropogenic activities have had disturbances and continue to exert an impact on portable water sources at the study area. Most of the groundwater sampled was found to be of potable quality except south zone of the city and this was mainly due to close proximity of sewage water treatment plant and groundwater sampled site. Some harvested rainwater sample also showed contamination of biofilm forming bacteria indicating the chances of potential risks to consumer's health. The study results would extremely enable the sanitary and health authorities to control by monitoring contamination of drinking water in Mysuru city. At the site of every overhead tank and storage tanks need to be disinfected regularly with periodic monitoring of drinking water quality at supply and storage sites. Provision of materials coated with metal or ceramic pipes to avoid microbial growth is necessary for the supply of safe potable water. Proper guidance at drilling and constructing wells, vigilant application of harvesting rainwater structures need to be implemented.

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