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Original Article

Comparative Evaluation of Thermotolerant *Escherichia coli*, Enterococci and Total Coliform as Indicators of Water Quality

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The present study was aimed to evaluate thermotolerant *Escherichia coli* and enterococci as alternative water quality indicator bacteria to assess the microbiological quality of surface water and supplied tap water of Dhaka city. Membrane filtration count of total coliform, *E. coli* and enterococci were obtained from various surface water bodies and supplied tap water. To find out the correlation of these indicators with the presence of water borne pathogens, counts of *Salmonella* and *Vibrio* were assessed concomitantly. The identifications of *E. coli*, *Salmonella* and *Vibrio* were performed by biochemical tests; whereas, for enterococci, identification by PCR method was applied. According to USEPA set rule for single sample analysis, the maximum concentration of d"235 *E. coli* or d"62 enterococci per 100 ml is considered a safe recreational or surface water standard for fresh water bodies. Following this, the present study found that out of the 22 surface water samples studied from 12 different surface water bodies in and around Dhaka city, 13 crossed the limit for *E. coli* and 10 crossed the limit for enterococci. On the other hand, all of the surface water bodies showed an exceedingly high number of total coliform bacteria regardless of the presence or absence of pathogens. Apart from only one sample, no correlation was observed between the presence of total coliform and the presence of the two pathogenic bacteria studied. On the other hand, *E. coli* and enterococci showed a better correlation in number with the presence of the pathogens; *E. coli* showing a better correlation than enterococci. Supplied tap water samples were examined from 8 different locations of Dhaka city. The international set rule is 0 enterococci or *E. coli* or total coliform or fecal coliform per 100 ml of potable water. All tap water samples showed considerable numbers of both total coliform and *E. coli* that always exceeded the limit allowed. However, out of the 8 samples, only 3 carried enterococci. *Salmonella* and *Vibrio* were obtained from most of the samples. In tap water the occurrence of *E. coli* showed more correlation with the presence of pathogens than with enterococci. This study suggested that for microbiological quality assessment of surface and tap waters of Dhaka city, total coliform has lost its credibility. Hence, *E. coli*, enterococci or other alternate water quality indicators suitable for subtropical region should be included in water quality testing.

Key words: Alternate water quality indicator bacteria, Thermotolerant *Escherichia coli*, *Enterococcus faecalis*, Total coliform

Introduction

Polluted water is a major environmental issue worldwide. In Bangladesh, about 80% of the disease outbreaks are waterborne. According to the British advocacy group WaterAID, Water-related diseases are responsible for 24 per cent of all deaths[1]. Every year, gastroenteritis and diarrhoeal diseases kill 110,000 children below the age of five. Over 4,100 children die from diarrhoea every year in Bangladesh and water-related diseases cause nearly a quarter of all deaths. Major waterborne diseases are cholera, bacterial and protozoal diarrhoea, hepatitis A and E, typhoid fever etc. Causes of this overwhelming incidence of waterborne diseases in Bangladesh can be attributed to the rudimentary water purification and sewage discharge systems, ignorance of the common people about proper waste and sewage disposal, traditional habit of using open field for excreta disposal and defecation. The matter of concern is, some underground water of Bangladesh that are collected through tube well has been reported to have coliform bacteria.

Faecal indicators are recommended in both temperate and tropical regions as microbiological water quality monitoring tools. However, various studies particularly in the tropics have expressed doubts in their reliability. This has been largely attributed to different conditions of the tropics like temperature, solar radiation, higher nutrient levels and the presence of a more diverse microbial community in the tropics. Various researches carried out in tropical countries question the appropriateness of using total coliform, fecal coliform and even *E. coli* (37°C) as indicator bacteria. An ever increasing number of studies have revealed that fecal bacteria, *E. coli* (37°C) and some pathogens such as *Salmonella, Shigella* and *Campylobacter*, have become naturalized to secondary habitats like soil, sands, sediments, water, phytoplanktons and zooplanktons[2-9]. The ubiquitous appearance

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of total and fecal coliforms in water samples of Bangladesh has necessitated the evaluation of other indicator bacteria.

In Bangladesh, total coliform and in a very few instances, fecal coliform bacteria are used as the only water quality indicators. Total coliforms are widespread in nature. All members of this group can occur in human feces, but some can also be present in natural habitats. Even fecal coliforms contain a genus, *Klebsiella*, with species that are not necessarily fecal in origin. High concentrations of organic materials can support bacterial populations, a portion of which are capable of responding positively to the total coliform and fecal coliform tests. Under the circumstances, the use of these traditional indicators is no longer justified in tropical waters. The present study aimed to assess the feasibility of thermotolerant *Escherichia coli*, *Enterococcus faecalis* and *E. faecium* as alternative indicator bacteria in Bangladesh. Surface water bodies in and around Dhaka city and supplied piped water were assessed to correlate the appropriateness of using different indicator bacteria.

Materials and Methods

Sampling sites

Surface water samples were collected from the following stagnant water bodies of lakes and ponds: Shahidullah Hall pond, University of Dhaka; Jagannath Hall pond, University of Dhaka; Bangla Academy pond; Mirpur pond; Sohrawardi Uddan pond; Dhanmondi lake; Gulshan lake; Mirpur lake and Dhaka Cantonment lake. Samples were also collected from the following rivers in and around Dhaka: the Buriganga river; the Shitolokkha river; the Turag river and the Brahmaputra river. Tap water samples were collected from the following areas of Dhaka city: Microbiology lab tap water, University of Dhaka; Shanir Akhra tap water; Sahidullah Hall canteen tap water, University of Dhaka; Jagannath Hall tap water, University of Dhaka; Agrani Bank, Dhaka University branch tap water; Mirpur area tap water; Cantonment area tap water; Uttara area tap water.

Sampling Procedure and Sample Transportation

The water samples were collected from relatively fresh flow and from a depth of 4.0 cm - 6.0 cm by pre-sterilized glass bottle or PET bottle. Standard procedures were followed for sampling[10]. Three representative samples were collected from each source aseptically. All samples were labeled at the spot and transported to the laboratory at the earliest convenience. Transportation to the laboratory was done in ice box in a temperature ranging from 4°C to 6°C. Microbiological tests were carried out as promptly as possible after collection to avoid unpredictable changes.

Membrane Filtration Method

Enterococci on Selective Media

Membrane filtration was carried out on EF medium, a selective and differential medium for *Enterococcus faecium and E. faecalis*. After incubation at 37°C for 48 hours *E. faecalis* forms pink or red brown colonies 0.5 – 2 mm in diameter, while *E. faecium* forms yellow colonies. Maroon colonies were carefully selected to obtain pure culture of *Enterococcus faecalis*.

Stock culture

Pure isolates were transferred into 1 ml nutrient broth in 1.5 ml eppendorf tube and grown overnight. Then glycerol was added to be stored at -70°C.

Microscopic examination for the identification of the isolates

The size, shape, arrangement, presence of endospore, staining properties, etc. of the vegetative cells of the selected strains were determined through microscopic examination.

Biochemical studies for identification of the isolates

Biochemical tests were performed according to the methods described in Manual of Methods for General Bacteriology by American Society of Microbiology[11].

Colony Polymerase chain Reaction (Colony PCR) of the isolated enterococci

One well isolated colony from fresh culture was taken in a PCR tube and mixed with 20 µl distilled water. This tube was incubated in the PCR thermocycler set at 100°C for 10 minutes for disintegration of the cells and allowing genomic DNA to be released from the cells. Cell debris was pelleted by centrifugation at 10,000 rpm for 5 minutes. The tube was cooled down to normal temperature and the supernatant was used as the sample DNA. PCR cocktails for 50µl reaction mixtures for each contained 2× reaction mixture containing 25µl premix taq, 1.2 µl forward primer, 1.2 µl reverse primer, 1µl sample DNA, 21.6 µl H_2O. PCR amplification was performed with 30 or 40 temperature cycles under standard conditions. At the end of the cycling steps a 10-min extension at 72°C was performed, and then samples were maintained at 4°C. Five µl of the PCR products was analyzed on 1% agarose gels. The PCR primers used were specific for enterococci and is described in table 1.

Table 1. *Primers used in the PCR method to amplify enterococci specific product*

Primer	Sequence (5´to 3´)	Tm (°C)	Amplicon size (bp)	Reference
ForwardEnt1	5¢-TACTGACAAACCATTCATGATG-3¢	55°C	112 bp	Ke *et al*., 1999[12]
ReverseEnt2	5¢-AACTTCGTCACCAACGCGAAC-3¢			

Result

Membrane filtration count

Table 2. *Relative number (c.f.u per 100 ml) of total coliform, thermotolerant E. coli, Enterococcus faecalis, Vibrio and Salmonella in six pond water samples around Dhaka University*

	Bacterial count (cfu/100ml)				
	Total coliform	*Thermotolerant E. coli*	*Enterococcus faecalis*	*Vibrio*	*Salmonella*
Shahidullah Hall pond sample 1	TNTC	250.0	400	480	0
Shahidullah Hall pond sample 2	TNTC	8.0	122	200	66
Jagannath Hall pond sample 1	TNTC	400.0	80	60	0
Jagannath Hall pond sample 2	TNTC	0.0	80	50	0
Bangla Academy pond	TNTC	600.0	50	140	10
Sohrawardi Uddan pond	TNTC	500	10	70	50

Table 3. *Relative number (c.f.u per 100 ml) of Total coliform, thermotolerant E. coli, Enterococcus faecalis, Vibrio and Salmonella in five water samples of Dhanmondi Lake, Dhaka*

	Bacterial count (cfu/100ml)				
	Total coliform	*Thermotolerant E. coli*	*Enterococcus faecalis*	*Vibrio*	*Salmonella*
Dhanmondi lake sample-1	TNTC	0	166	82	44
Dhanmondi lake sample-2	TNTC	4	144	40	80
Dhanmondi lake sample-3	TNTC	40	42	12	26
Dhanmondi lake sample-4	TNTC	30	26	8	20
Dhanmondi lake sample-5	TNTC	50	40	10	0

Table 4. *Relative number of Total coliform, thermotolerant E. coli, Enterococcus faecalis, Vibrio and Salmonella in five lake water samples of Dhaka City*

	Bacterial count (cfu/100ml)				
	Total coliform	*Thermotolerant E. coli*	*Enterococcus faecalis*	*Vibrio*	*Salmonella*
Mirpur Lake -1	TNTC	TNTC	TNTC	400	120
Mirpur Lake -2	TNTC	TNTC	44	TNTC	180
Cantonment lake -1	TNTC	500	46	TNTC	0
Cantonment lake -2	TNTC	500	TNTC	TNTC	0
Gulshan lake	TNTC	TNTC	0	46	0

Table 5. *Relative number of Total coliform, thermotolerant E coli, Enterococcus faecalis, Vibrio and Salmonella of four river water in and around Dhaka City*

	Bacterial count (cfu/100ml)				
	Total coliform	*Thermotolerant E. coli*	*Enterococcus faecalis*	*Vibrio*	*Salmonella*
Turag sample-1	TNTC	TNTC	12	29	21
Turag sample-2	TNTC	TNTC	40	32	20
Brahmaputra sample-1	TNTC	44	22	25	20
Brahmaputra sample-2	TNTC	38	24	32	21
Shitolokkha	TNTC	TNTC	400	20	10
Buriganga	TNTC	TNTC	TNTC	50	10

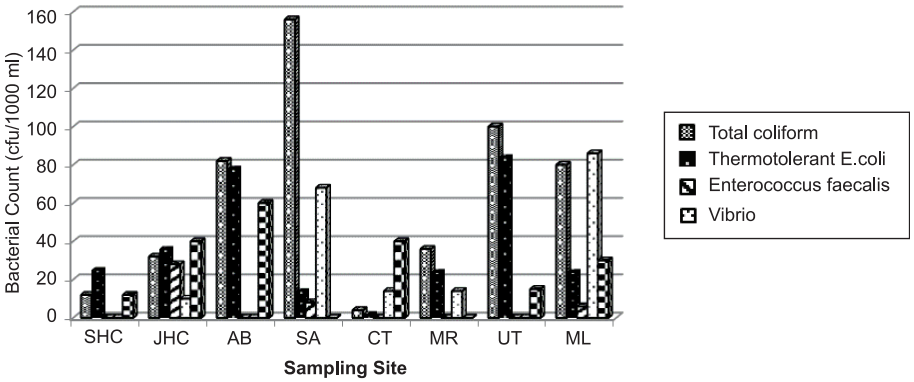

Figure 1. *Relative number (c.f.u per 100 ml) of Total coliform, thermotolerant E coli, Enterococcus faecalis, Vibrio and Salmonella of eight tap water sampls of Dhaka City. SHC Shahidullah Hall Canteen; JHC Jagannath Hall canteen; AB Agrani Bank DU; SA Shanir Akhra; CT Cantonment; MR Mirpur; UT Uttara; ML Microbiology Lab*

PCR results for identification of enterococci

PCR amplification products of 112 bp of enterococci specific gene were obtained which is shown in Figure 2. The confirmation of *Enterococcus faecalis* was done by combined results of these PCR, the highly specific maroon to deep red colonies of 0.5-2 mm dia on EF agar plate (Figure 3) and also the cell morphology and arrangements in Gram staining (Figure 4).

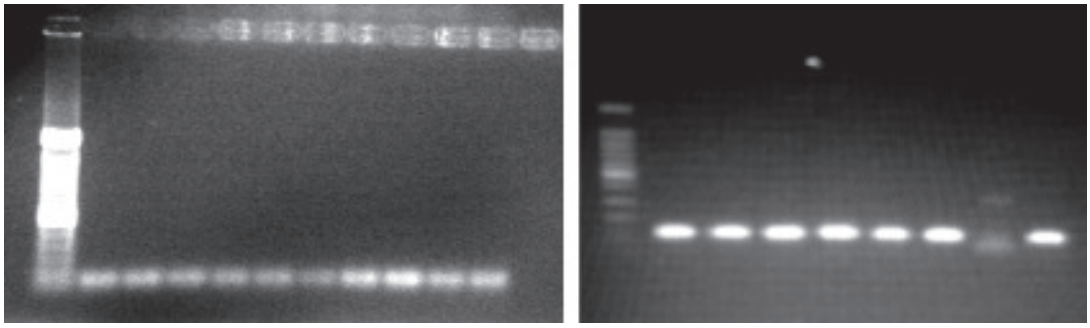

Figure 2. *PCR amplification product of enterococci. A 112 bp PCR amplification product was obtained which is specific for enterococci. The molecular weight marker seen on the left lane is 100 bp DNA marker (NEB, UK)*

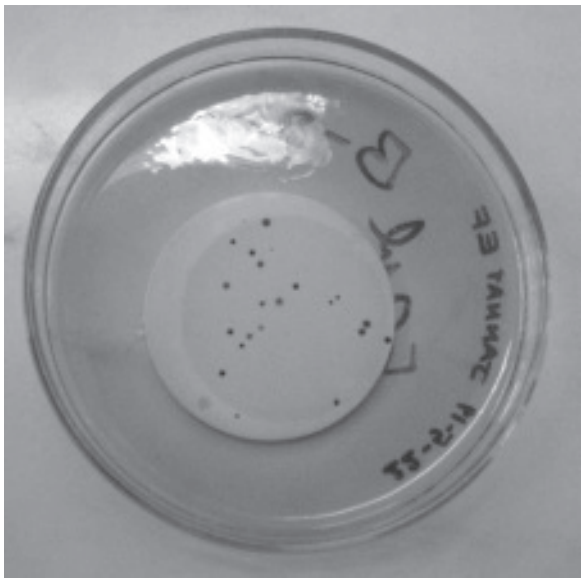

Figure 3. *Maroon colonies of Enterococcus faecalis on EF agar*

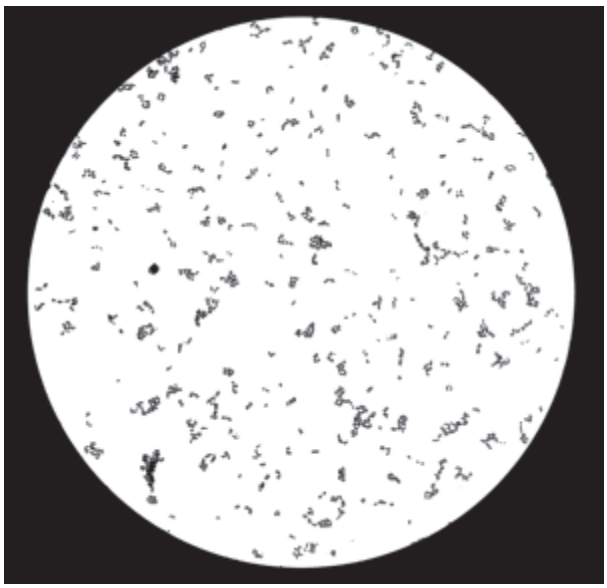

Figure 4. *Cell morphology and arrangement of Enterococcus faecalis under microscope*

Discussion

In the tropics, the classical indicators of water pollution are suspected to originate from non-faecal sources (such as soil) and to proliferate in tropical aquatic habitats under favourable situations and thus can be detectable at levels which may not reflect the original extent of faecal contamination[2-9].

The enterococcus group includes two strains of the fecal streptococci that are human specific, namely, *Streptococcus faecalis* and *Streptococcus faecium*. Studies on marine and fresh water bathing beaches indicated that swimming-associated gastroenteritis was related directly to the quality of the bathing water and that enterococci were the most efficient bacterial indicator of water quality[13,14]. *Streptococcus faecalis* has the advantage over *E. coli* in that it survives better in the aquatic environment.

Many members of the total coliform group and some so-called faecal coliforms (e.g. species of *Klebsiella* and *Enterobacter*) are not specific to faeces, and even *E. coli* has been shown to grow in some natural aquatic environments[8,15-19]. Hence, the primary targets representing faecal contamination in temperate waters are now considered to be *E. coli* and enterococci.

The complexity of the tropical environments in relation to the expectable indicator performances has not been taken into consideration in our country so far. We have continued accepting and relying on guidelines established for temperate regions. Therefore, in many instances, the bacterial indicators that are currently in use do not represent the actual number of fecal bacteria in the water samples giving false positive results. There is thus scope and need to reassess, evaluate and explore existing novel methods and parameters for fecal contamination monitoring in the tropical waters. Hence, the present study was undertaken to assess microbiological qualities of several surface water bodies and supplied tap water of Dhaka city and to make a comparative assessment of the credibility of the traditional water quality indicators like total coliform with thermotolerant *E. coli* and enterococci. The presence of enterococci is a valuable bacterial indicator for determining the extent of fecal contamination of recreational surface waters[20].

Indicators vary in their ability to reliably predict potential risks to human health. Some indicators have been shown to have a greater statistical relationship to disease than others. Also, current indicators are based on fecal contamination and might not accuratelty assess the potential for disease from other pathogens that can cause skin, upper respiratory tract, eye, ear, nose and throat disease[21]. More research on the use of other bacteria and viruses as indicators is being conducted at the federal, state, and local levels in the USA. Despite variability in the ability of indicators to reliably predict potential risks to human health, EPA studies indicate that enterococci and *E. coli* are the most effective available primary indicators for predicting the presence of gastrointestinal illness-causing pathogens, and for marine waters, enterococci is most appropriate.

One area of current scientific debate is whether indicator bacteria react differently under various climatic and environmental conditions. Preliminary evidence suggests that *E. coli* and enterococci can be detected at tropical locales such as Puerto Rico, Hawaii, and Guam in waters where there is no apparent source of contamination from warm-blooded animals[21]. EPA and others are evaluating whether the current indicator bacteria grow and persist in natural tropical environments.

Historically, fecal coliforms and *E. coli* have been used as indicators of choice when monitoring recreational water quality[22]. Recent studies have shown that high densities of *E. coli* and enterococci recovered from recreational waters have a stronger correlation with swimming-associated gastrointestinal disease than do densities of fecal coliform bacteria[23]. Although enterococci have been traditionally used to monitor marine bathing water[24], both of these indicators have been referenced as being equally acceptable for monitoring freshwater[22,25]. Therefore, studies of both marine water and freshwater have been undertaken to support the idea that enterococci may be the more relevant indicator of water quality[22].

The abundance of enterococci in human and animal feces, the ease with which they are cultured, and their correlation with human health outcomes in fresh and marine waters have led to their widespread use as tools for assessing recreational water quality worldwide[13,26-29]. However, many reports also state that *E. coli* should be the indicator of choice for fresh water quality[30].

Previous studies have shown that *E. coli* (37°C) can become naturalized to the microbial community in tropical, subtropical, and temperate soil and sand[31,32,33]. This likely limits the use of this bacterium as an indicator of water quality. Moreover, these culture-based methods cannot differentiate among sources of fecal bacteria[34].

It has been proved that the conventional indicators of fecal origin i.e. coliform bacteria (total and fecal coliforms), used to evaluate microbiological quality of waters provide erroneous information[35]. They do not adequately reflect the occurrence of pathogens in disinfected wastewater effluent due to their relatively high susceptibility to chemical disinfection and failure to correlate with protozoan parasites and enteric viruses[36]. As well as, coliforms are generally considered unreliable indicators of faecal contamination because many are capable of growth in the environment. Thus, the public health is not protected by using these common indicators such as total coliform and fecal coliform.

The present study was an effort to verify the authenticity of using total coliforms as surface water quality indicators in a tropical country like Bangladesh and also to verify the feasibility of *E. coli* and *Enterococcus faecalis* as an alternative water quality indicator. It was observed that in all the surface water samples tested, the total coliform exceeded the countable limit beyond the allowed limit, even in the water bodies where faecal contamination was not that obvious. Moreover, total coliform numbers did not show any correlation with the numbers of the two waterborne pathogens tested, namely *Vibrio* and *Salmonella*

spp. This justifies the statements of many previous findings that stated these indicators are becoming acclimatized to and proliferate in the tropical water bodies. Thermotolerant *E. coli* (45°C) and *Enterococcus faecalis,* on the other hand, showed a correlation with the presence of these two pathogens. Their number was always within a countable limit, and always exceeded the number of pathogens suggesting their validity as a more acceptable surface water quality indicator in our country. Different countries and different organizations have set different allowable limits of different indicators. The present data were analyzed taking the limit of USEPA[37] for single count measurement for fresh recreational water, which considers 62 cfu / 100ml to be the highest permissible limit for enterococci, and 235 cfu / 100 ml for *E. coli*. At present the USEPA does not consider total coliform as a recreational water quality indicator any more.

In all the samples (except S2) studied, total coliform always exceeded the permissible limit showing matt growth on the membrane filter, even in diluted samples. It showed no correlation with the presence of *Vibrio* and *Salmonalla*. On the other hand, *E. coli* and *Enterococcus faecalis* showed a much better correlation with the presence of these pathogens in all the samples studied.

Among the stagnant water bodies studied, Dhanmondi lake was apparently the cleanest. This observation correlated with the findings showing much less number of both *E. coli* and *Enterococcus faecalis* as well as of the pathogens (Table 3). Other lakes and ponds showed quite a high number of these indicators and in some crossing the recommended limit. Mirpur and Gulshan lakes showed a very high number of *Vibrio* as well as *E. coli* and *Enterococcus faecalis* (Table 4). Amongst the river water studied, Brahmaputra and Shitalakhya were the samples outside Dhaka. Brahmaputra water sample was collected from Mymensingh and the river is apparently the cleanest of all. This organoleptic observation was reflected in the results as well showing the least number of *E. coli* and *Enterococcus faecalis* as well as the pathogens (Table 5). The Shitalakhya and the Buriganga rivers showed a very high number of *Vibrio* as well as the indicators *E. coli* and *Enterococcus faecalis* (Table 5). Both the water samples of the Turag river showed a very high number of *E. coli.* However, enterococci did not cross the limit in these two samples (Table 5) The tap water samples crossed the limit of *E. coli* and total coliform in most of the samples (Figure 1). *Salmonella* and *Vibrio* were present as well. The tap water samples from various sites of Dhaka city were tested along with the surface waters, to observe the numbers of these three indicators so that the authenticity of these indicators can be compared and justified. According to established guidelines of WHO, USEPA enterococci and *E. coli* should not be present in any 100 mL sample of raw drinking water. Our pipeline waters are treated by chlorination, still they possessed both of the indicators and sometimes pathogens as well. However, only a very few of the tap water samples showed the presence of *Enterococcus faecalis.* Cross contamination of the water supply lines with sewage or drain water is likely to occur. However, the total and thermotolerant *E. coli* were always present in these tap water samples. In this case, however, thermotolerant *E. coli* correlated more with the presence of the pathogenic bacteria. It might seem unrealistic why coliforms are present in supplied treated piped water. Unfortunately, this is the reality in Bangladesh. Many previous and current research papers show similar statistics of the presence of total coliform, faecal coliform and even *Salmonella*, *Shigella* and *Vibrio cholera* in supply water of Dhaka[38,39,40]. In such a context, the supplied piped water of Dhaka city cannot be certified to be safe for drinking without prior treatment. Although, except for the slum dwellers, people of Dhaka city do not directly drink supplied piped water without treatment, it is the duty of concerned authority to provide absolutely safe drinking water through the pipeline. At least the bacterial load and indicator load must be lowered down to an acceptable limit. The number of total coliform, thermotolerant *E. coli*, *Enterococcus faecalis*, *Salmonella* and *Vibrio* that the present study found out indicated a very high level of harmful disease causing bacteria in the supplied drinking water which is not at all acceptable under any circumstances. Mahbub *et al*.[40] reported that among the 45 piped water samples they studied, 57.78% samples exceeded the BDS standard and WHO guideline for coliform bacteria and 51.11% for *E. coli* bacteria. Total Coliform and *E. coli* count in water samples ranged from <1.8 to >1600(MPN) / 100 ml. These values for Total coliform and *E .coli* are unacceptable for drinking water[41]. They also found that most of the pump water of WASA which use deep tube well is free of bacterial load and the highest amount was found in house tap water. They concluded that the source of contamination mainly is the distribution system of water of Dhaka city. Therefore, quality of pipelines, integrity of pipes and junctions between pipes, proper and adequate chlorination must be checked and maintained at a regular basis. Otherwise, this poor quality of piped water cannot be upgraded. There is a conception among the city dwellers that the piped water is not meant for drinking purpose, which is a wrong concept. Unfortunately, their conception about the health risk for drinking piped water is correct. Our piped water must be made safe for drinking. This is possible as it does not require a much investment. It is necessary to monitoring piped water at the treatment point and at different locations of the city regularly and taking adequate corrective measures wherever any contamination source is observed.

Conclusion

It is seen from the results of this study that, both thermotolerant *E. coli* and enterococci showed a correlation with the presence of *Vibrio* and *Salmonella* in various fresh surface water bodies. However, thermotolerant *E. coli* showed better correlation. Total coliform number exceeded the upper limit set for recreational water bodies and did not correlate with the pathogen numbers. All the tap water samples crossed the limit (which is 0 per 100 ml) of *E.*

coli and total colifom. However, only 3 samples (JHC, SA, ML) crossed the limit for enterococci (Figure 1). Most of the samples had *Salmonella* and *Vibrio*. Hence, the supplied piped water must be made drinkable for the sake of public health. The level of indicators as well as pathogens isolated from piped water from different areas of Dhaka city is not at all acceptable.

The appropriate concerned authorities of Bangladesh must include alternative water quality indicators (enterococci, thermotolerant *Escherichia coli*) as well as some suggested pathogenic bacteria in their routine survey and assessment of water quality testing, as it is quite apparent from this and some other previous studies that the validation of still using total coliform as water quality indicator has become questionable.

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