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REVIEW ARTICLE

Microbiological approach to enumerate microbes and pathogens in drinking water samples

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ABSTRACT
The effects of drinking contaminated water lead to thousands of deaths a day. Consumption of water contaminated with infective microorganism is usually accountable for the onset of waterborne disease outbreaks, particularly in developing countries. Such infected water might cause several unwanted diseases like typhoid, cholera, acute diarrheal diseases, etc. Consumption of contaminated water and poor hygiene practices are the leading reason for death among kids worldwide, after respiratory diseases. E. coli, Coliforms, Streptococci, and Enterococci bacteria act as indicator organisms of water contamination. This review discusses and summarizes microbiological approach and methodologies for the evaluation of normal microbial flora and pathogens in the drinking water samples which indicates their potability for consumption.
***Key words**: Drinking water, Coliforms, Potable, Pathogens*

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INTRODUCTION

Drinking water is one of the foremost essential components of our life and it is also very closely related to human health [1]. Since, the routine examination of water samples from various sources for the presence of harmful pathogens is often tedious, difficult and time-consuming task and therefore it is customary to examine the water samples by looking for such microorganisms whose presence indicates that pathogens might be present. These bacteria can easily be isolated and quantified by simple bacteriological methods. Detection of these bacteria in water means that faecal contamination has occurred and suggest that enteric pathogens may also be present [2].

About 33% of morbidity occurs because of the intake of poor-quality water worldwide and most of the other diseases also are linked with water [3]. Around 4.6 billion of diarrheal cases were estimated over the planet by WHO, which caused the death of 2.2 million people of whom majorities were children below five years [4]. Most of the bacterial diseases are transmitted through the water like cholera, typhoid and dysentery. The presence of pathogens in water is most vulnerable and it's quite impossible to examine the water for all known waterborne pathogens to suggest whether it's safe for drinking or not [5]. Microbiological water analysis is especially based on the concept of faecal indicator bacteria [6]. Safe water for all is one amongst the most important challenges of the 21st century and also the microbiological control of water should be the norm everywhere. Routine basic microbiological analysis of water should be carried out by assaying the presence of *E. coli* by culture methods [7]. Morbidity resulting from the ingestion of contaminated water persists globally, and the available epidemiological evidence demonstrates a dramatic increase in the number of waterborne outbreaks and individual cases [8].

Coliform bacteria, which usually exist within the intestines of almost all the warm-blooded animals, are passed out in considerable amounts in the faeces [9]. In contaminated water, coliform bacteria are based in solidity approximately comparable to the extent of faecal contamination. Since coliform bacteria are usually sturdy than disease-causing bacteria, their non-existence from water is a sign that the water is bacteriologically fit for human use [10]. Contrarily, the existence of the coliform group of bacteria is a

symbol that different sort of microorganism is capable of inducing disease and that the water is certainly unfit to consume [11].

Indicators have conventionally been utilized to demonstrate the existence of enteric pathogens; anyhow, nowadays we acknowledge that there is hardly ever a straight connection among the bacterial indicators and human pathogens [12]. As such, the use of indicators is preferably elucidated by their intentional aim. Therefore, process indicators are utilized to evaluate the effectiveness of a treatment procedure (e.g., drinking water treatment), while faecal indicators show the existence of faecal pollution. An index (or model) organism stands for the existence and behavior of a pathogen in a given environment [13].

In 1914 the U.S. Public Health Service adopted the coliform group as an indicator of faecal contamination of drinking water [14]. Many countries have adopted coliforms and other groups of bacteria as official standards for drinking water, recreational bathing waters, wastewater discharges, and various foods. Indicator microorganisms have also been used to assess the efficacy of food processing and water and wastewater treatment processes [15]. As an ideal assessor of faecal contamination, it has been suggested that they meet the criteria listed in Table 1.

Table 1.Criteria for an Ideal Indicator Organism

S. No.	Criteria
1	The organism should be useful for all types of water
2	The organism should be present whenever enteric pathogens are present
3	The organism should have a reasonably longer survival time than the hardiest enteric pathogen
4	The organism should not grow in water
5	The testing method should be easy to perform
6	The density of the indicator organism should have some direct relationship to the degree of faecal pollution
7	The organism should be a member of the intestinal microflora of warm-blooded animals

Checking water for all tenable microorganisms is difficult, slow, and costly [16]. It is comparatively uncomplicated and low-priced to investigate for coliform bacteria. If coliform bacteria are found in a water sample, water system operators work to find the source of contamination and restore safe drinking water [17]. There are three different groups of coliform bacteria, each incorporates a different level of risk. Total coliform, faecal coliform, and *E. coli* are all indicators of drinking water quality [18]. Total coliform group is a large collection of various types of bacteria. Faecal coliforms are sorts of total coliform that mostly exist in faeces. *E. coli* is a sub-group of faecal coliforms [19]. Specifically, this group includes all aerobic and facultatively anaerobic, gram-negative, spore-forming, rod-shaped bacteria that produce gas upon lactose fermentation in prescribed culture media.

The coliform group has been used as the standard for assessing faecal contamination of recreational and drinking waters since early in the twentieth century. Total coliform bacteria are commonly found within the environment (e.g., soil or vegetation) and are generally harmless. If only total coliform bacteria are detected in drinking water, the source is probably environmental [20]. Faecal coliform bacteria are a sub-group of total coliform bacteria. They appear in great quantities within the intestines and faeces of individuals and animals. The presence of faecal coliform in a potable water sample many a time indicates recent faecal contamination, expressing that there is a huge risk that pathogens are present than if only total coliform bacteria is found [21]. *Escherichia coli*, a member of the coliform group, is sometimes considered the foremost vulnerable coliform bacteria since its presence indicates faecal contamination and also as the possibility of enteric disease [22]. *E. coli* are regularly found in the various natural environment as well as in the intestine of human but not in drinking water because the survivability of *E. coli* in drinking water at around 16 ºC is only 4-12 weeks [23].

Table 2. Guidelines for determination of faecal contamination of water

Class	Grade of water sample	Presumptive coliform count/ 100 ml	*E. coli* count per 100 ml
I	Excellent	0	0
II	Satisfactory	1–3	0
III	Suspicious	4–10	0
IV	Unsatisfactory	> 10	0, 1 or more

Source- WHO, Second Edition, Vol. III, 1997

Four widely used standard enumeration methods for faecal coliform in drinking water are multiple tube fermentation technique, gene probe test, the heterotrophic plate count, and the membrane filtration techniques that provides the count of colony-forming unit per 100 ml when placing on the surface of agar after filtration of liquid medium [24, 25]. The maximum acceptable number of *E. coli* in portable drinking water should be none per 100 ml [26]. The Multiple-Tube technique is also expressed as Most Probable Number (MPN) test allows detection of the presence of coliforms in a sample and estimation of their numbers. The technique is employed extensively for drinking-water analysis, but it's time-consuming to perform and requires more equipment, glassware, and consumables than membrane filtration. However, the multiple tube method may be more sensitive than membrane filtration [27]. This test consists of three steps: a presumptive test, a confirmed test, and a completed test. In the presumptive, lauryl sulfate–tryptose–lactose broth is put in some test tubes with various solutions of the water to be evaluated. Generally, four to five test tubes are made ready per dilution [28]. These test tubes are incubated at 35°C for 24 to 48 h and then inspected for the existence of coliforms, which is denoted by gas and acid output. When the positive tubes have been recognised and documented, it is likely to guess the total number of coliforms in the native sample by utilising the Most Number Probable table that provides numbers of coliforms per 100 ml [29]. In the affirming test, the existence of coliforms is confirmed by inoculating selective bacteriological agars like Levine's Eosin–Methylene Blue (EMB) agar or Endo agar with a little quantity of culture from the positive tubes. Lactose- fermenting bacteria are specified on the medium by the yield of colonies with a green lustre or colonies with a dark centre [30]. In a few instances, a concluded trial is carried out in which colonies from the agar are inoculated back into lauryl sulfate–tryptose–lactose broth to illustrate the output of acid and gas. The Membrane Filtration trial further let the scientists decide the number of coliforms in a sample, however, it is simple to carry out Membrane Filtration than the Most Probable Number test as it requires lesser test tubes and few labours [31]. In this approach, a dignified volume of water (usually 100 ml for drinking water) is transferred by a membrane filter (pore size 0.45 μm) that catches bacteria on its surface. This membrane is then put on a fine absorbent pad that has been suffused with a particular medium intend to allow development and differentiation of the organisms being called on. For example, if total coliform organisms are sought, a reconstructed Endo medium is utilised. For coliform bacteria, the filter is incubated at 35°C for 18–24 h[32]. The triumph of the procedure relies on using effectual differential or selective media that can facilitate recognition of the bacterial colonies developing on the membrane filter surface. To check the number of coliform bacteria within a water sample, the colonies having a green lustre are calculated [33]. Gene probes are fragments of DNA that search out and gets attached with cDNA fragments. Often the test is designed to test for the presence of *Escherichia coli* in water [34]. To use a gene probe technique for *E. coli* in water, the water is made to disrupt any bacterial presence. Then a particular *E. coli* probe is put into the water like a left seeking a right, the probe searches through all the nucleic acid within the water and unites with the *E. coli* DNA if present. A radioactive signal specifies that a match has been created. If no radioactivity is released, then the probe has been unable to find its matching DNA, and *E. coli* is likely to be missing from the water [35]. It is generally impractical to test for all pathogenic organisms, but the total number of bacteria can be calculated. One test is the standard plate count. In this trial, samples of water are mixed in jars containing 99 ml sterile water, and samples are put in Petri dishes with agar or a different wholesome medium. After incubation, the colony count is taken and multiplied by the dilution factor to obtain the total number of bacteria per ml of sample [36]. The membrane filter technique uses a filtration apparatus and a cellulose filter called a membrane filter [37]. A 100 ml sample of water is skilled the filter, and therefore the filter pad is then transferred to a bacteriological growth medium. Bacteria trapped within the filter grow on the medium and form colonies. By counting the colonies, an estimate can be made of the number of bacteria in the original 100 ml sample [38]. An evaluation of the figures of aerobic and facultative anaerobic bacteria within the water that gains their carbon and vitality from living

compounds is managed through the heterotrophic plate count or HPC [39]. This category comprises of gram-negative bacteria which is a member to the following genera: *Pseudomonas, Aeromonas, Klebsiella, Flavobacterium, Enterobacter, Citrobacter, Serratia, Acinetobacter, Proteus, Alcaligenes, Enterobacter, and Moraxella* [40]. The above-mentioned bacteria are often segregated from surface waters, groundwater and are globally in soil and plants. Various members of this category are opportunistic pathogens (e.g., *Aeromonas, Pseudomonas*), but no undeniable proof is obtainable to show their transference through drinking water [41]. In drinking water, the number of heterotrophic plate count bacteria might differ from less than 1 to more than 104 CFU/ml, and they are affected mostly due to temperature, the existence of remaining chlorine, and level of assimilable living matter. In actuality, these counts themselves have nil or minor health significance. However, there has been a concern because the heterotrophic plate count can grow to large numbers in bottled water and charcoal filters on household taps. In response to this concern, studies have been performed to evaluate the impact of heterotrophic plate count on illness [42]. Researchers have not shown an undeniable effect on the disease in humans who drink water with high heterotrophic plate count. Even though the heterotrophic plate count is not a straight measure of faecal pollution, it does specify variety in water standard and the ability for pathogen survival and regrowth. These bacteria might further obstruct with coliform and faecal coliform identification when they exist in high numbers. It has been proposed that the heterotrophic plate count shall not pass 500 per ml in tap water [40]. Heterotrophic plate counts are generally carried out by the spread plate method utilising the yeast extract agar incubated at 35°C for 48 h. A less-nutrient medium, Reasoner's 2A agar (R_2A),has seen global use and is advised for disinfectant-damaged bacteria [43]. This medium is recommended for use with an incubation period of 5–7 days at 28°C. Heterotrophic Plate Count (HPC) numbers can differ significantly relying on the temperature of incubation, growth medium, and incubation length. Even though the total coliform category has performed as the foremost measure of water contamination for countless years, numerous organisms in this class are not restricted to faecal sources. Therefore, techniques have been evolved to limit the enumeration to coliforms that are more clearly of faecal origin—that is, the faecal coliforms. These microorganisms, which comprise of the genera *Escherichia and Klebsiella*, are transformed in the laboratory by their capability to ferment lactose with the manufacturing of acid and gas at 44.5°C within 24 h [44]. In general, this test indicates faecal coliforms; it does not, however, distinguish between human and animal contamination. The repeated existence of coliform and faecal coliform organisms in disinfected tropical waters, and their expertise to live for substantial time period exterior to the intestine in these waters, have suggested that these organisms occur naturally in tropical waters and those new indicators for these waters need to be developed [45]. Some have suggested the use of *E. coli* as an indicator because it can easily be distinguished from other members of the faecal coliform group (e.g., absence of urease and presence of β- glucuronidase) and is more likely to indicate faecal pollution [46]. Faecal coliforms also have some of the same limitations in use as the coliform bacteria (i.e., regrowth and less resistant to water treatment than viruses and protozoa).Faecal coliforms might be identified by the techniques like those employed for coliform organisms. For the Most Probable Number method, EC broth is used, and for the membrane filter method, m-FC agar is used for water analysis. A medium known as m-T7 agar has been proposed for use in the recovery of injured faecal coliforms from water and results in greater recovery from water [47]. The Colilert trial has the benefit of identifying coliforms and *E. coli*, the main faecal coliform, concomitantly within 24 h. The faecal streptococci belong to the genera *Enterococcus* and *Streptococcus*.[48]. The genus *Enterococcus* includes all streptococci that share certain biochemical properties and have a wide range of tolerance of adverse growth conditions. Out of the genus *Streptococcus* which is frequently provided by the water industry, wholly *S. bovis* and *S. equinus* are accounted to be real faecal streptococci [49]. The above mentioned two species of Streptococcus are mostly discovered in animals. It has been proposed that a faecal coliform/faecal streptococcus (FC/FS) ratio of 4 or more specifies pollution of an individual start, considering that a ratio below 0.7 is symptomatic of animal contamination as shown in Table 3. Although, the authenticity of the FC/FS ratio has been challenged. Moreover, this ratio is viable only for a recent (24 h) faecal contamination.

Table 3. The FC/FS Ratio

FC/FS Ratio	Source of Pollution
>4.0	Strong evidence that pollution is of human origin.
2.0-4.0	Good evidence of the predominance of human wastes in mixed pollution.
0.7-2.0	Good evidence of the predominance of domestic animal wastes in mixed pollution.
<0.7	Strong evidence that pollution is of animal origin.

Both, the Membrane Filtration technique, as well as Most Probable Number technique, can also be utilized for the segregation of faecal streptococci. The Membrane Filter technique utilises the faecal Streptococcus agar with incubation at 37°C for 24 h. All Maroon, Pink, and Red colonies are counted as tentative faecal streptococci. Assurance of faecal streptococci is through the subculture on bile aesculin agar and incubation for 18 h at 44°C. Faecal streptococci form separate colonies enclosed by a Black or Brown radiance because of aesculin hydrolysis and *Ent. faecalis* are regarded as to be much more particular to the individual gut. Faecal streptococci are believed to have some benefit with reference to the coliform and faecal coliform organism as indicators. The enterococci have been recommended as a helpful index of the possibility of gastroenteritis for jolly bathers and further standards have also been recommended [50].

DISCUSSION

A wide array of indicator organisms has been proposed for brackish, estuarine and coastal waters. It is clear that no single indicator, index or reference organism exists for tittering public health safety of these waters. Thus, different microbes are used as indicator organisms for analysis of water as shown in Table 4. The growth medium and methodology for the enumeration of normal microbes and pathogens are described in Table 5 and 6. These pathogens are selective and cause serious illness and diseases as shown in Table 7. However, developments in biotechnology such as immuno-epifluorescent microscopy technique, use of monoclonal antibody for detection of pathogens have made a significant contribution to assure the public health safety. The advent of gene probes will also be potential in the detection of toxin gene carrying microorganisms in water and other samples from the aquatic environment. Reports of Streptococcal durability in the environment w.r.t other indicators are often inconclusive or contradictory. However, most studies have shown that faecal streptococci outlive coliforms and faecal coliforms in effluents and aquatic environments.

Epidemiological studies conducted by the U.S Environmental Protection Agency (USEPA) have shown that *Enterococcus* concentrations are better correlated than faecal coliforms concentration with the disease risk associated with sewage polluted water.Chowdhory Arman *et. al*,in the year 2016, discussed in their paper about the detection of *Escherichia coli* in drinking water sources of filter units and supply water. Percival Steven *et. al,* in the year 2000, reviewed the development of biofilms and their role drinking water-related issues. They review methods for studying the epidemiological spread of waterborne infections. McFeters Gordon in the year 2013 described the microbiology of the drinking water. He also discussed the incidence of waterborne outbreaks of unknown aetiology and those caused by 'new' pathogens such as Campylobacter sp. Cabral J.P in the year 2010, reviewed a general characterization of most important bacterial diseases transmitted through water, focusing on the biology and ecology of the causal agents. Emerging pathogens and the importance of pathogenic *Escherichia coli* strains in drinking water diseases are also briefly discussed. Geldreich Edwin [20] suggested that official monitoring of small water supplies must be increased on a monthly basis and inform water plant operators of unsatisfactory water qualities. Further, the quality of water in terms of chlorine residual, turbidity, total coliforms, etc. should be analyzed. Holinger Eric *et. al,* in the year 2014, have analyzed the bacterial compositions in the tap water and found that *Proteobacteria (35%), Cyanobacteria (29%, including chloroplasts), Actinobacteria* (24%, of which 85% were *Mycobacterium* sp. Were present. The genus Mycobacterium was most abundant taxon in the dataset, detected in 56 out of total 63 samples. Edberg Stephen *et. al*, [7] discussed a defined substrate method that was developed to simultaneously enumerate total coliforms and *Escherichia coli* from drinking water without the need for confirmatory or completed tests. If total coliforms are present in the water sample, the solution will change from its normal colourless state to yellow.

Table 4.Showing different indicator organisms commonly used for the analysis of water

Organism	Disadvantage	Significance	CFU per 100 ml
Coliforms	• Regrowth in aquatic environments • Regrowth in distribution systems • Suppression by high background bacterial growth	• Poorly correlated with gastrointestinal disease.	10^7 –10^9
Faecal coliforms	• Presence of bacterial pathogens which are unrelated to human waste	• It selects coliform of faecal origin by using a higher incubation temperature.	10^6 –10^7
Faecal streptococci	• Sensitivity to UV radiations is still not clear. • Epidemiological studies are difficult and expensive to carry out.	• Persistence without multiplication in the environment. • Absence from pure water and having no contact with human and animal life.	10^5–10^6
Enterococci	• Their determination is time-consuming and unreliable.	• High survival rate.	10^4–10^5
E. coli	• Difficulty in isolation of *E. coli*	• More significant as an indicator in water quality than faecal coliforms test.	10^2 –10^3

Table 5. Growth medium and methodology utilized for enumeration of normal microbes in drinking water samples

S. No.	Name of microorganisms present in water	Media used	Name of the method	Reference
01	Coliforms	MacConkey agar	Colilert test	**58**
02	Spore-Formers	Mannitol Egg Yolk Polymyxin	Enzyme-Linked ImmunoSorbent Assay (ELISA)	**59**

Table 6. Growth medium and methodology utilized for enumeration of pathogens in drinking water samples

S. No.	Name of pathogens	Media used	Name of the method	Reference
01	*Yersinia enterocolitica*	MacConkey agar	Indole test	52
02	*Salmonella typhi*	Bismuth sulfite agar	Normal Flow Filtration (NFF) and Tangential Flow Filtration (TFF)	**53**
03	*Vibrio cholerae*	Thiosulfate-citrate-bile-salts-sucrose agar	Phenol-Chloroform method	54
04	*Escherichia coli.* (specific strains)	Membrane filter agar	DipTest: Litmus paper test	55
05	*Shigella dysenteriae*	Hektoen enteric agar	PCR and culture techniques	56
06	*Clostridium perfringens*	Differential and selective broth	Membrane filtration using microporous membranes typically composed of cellulose esters	**57**

Table 7. Pathogens in water and their relevant diseases

S. No.	Name of pathogens	Disease caused by the pathogens	Reference
01	*Yersinia enterocolitica*	Gastroenteritis	51
02	*Salmonella typhi*	Typhoid fever	
03	*Vibrio cholerae*	Cholera	
04	*Escherichia coli.* (specific strains)	Gastroenteritis	
05	*Shigella dysenteriae*	Bacillary Dysentery	
06	*Pseudomonas aeruginosa*	Various Infections	

CONCLUSION

The purposes of this work were to compile an inventory of traditional and more recent coliform group detection methods, mainly those applied to the analysis of the microbiological quality of drinking water. Today, the Membrane Filtration technique is the most widely used method for the enumeration of coliforms in drinking water. This technique is simple to perform and is inexpensive, and it also requires at least an overnight incubation period and a confirmatory test (24 to 72 additional hours) after the initial typical colony investigation. Furthermore, when standard agar media are used with this technique, it is not possible to recover stressed or injured coliforms. Specially developed media and the addition of specific compounds improve the recovery rate of these stressed or injured cells. The main problem for the growth of new coliform identification techniques is to upgrade the specificity of the technique, which may remove the time-consuming assurance stage, to take into consideration, stressed and wounded cells and to lower the investigation time.

A particular enzymatic activity analyzation should upgrade the susceptivity of coliforms and *E. coli* identification, using β-D galactosidase and a β-D glucuronidase. Numerous chromogenic and fluorogenic substances occur for these enzymatical identification activities, and different commercial trials on the grounds of these substances are accessible. Numerous comparisons between these tests and the standard methods have shown that they may be a suitable alternative to the Membrane Filtration technique. The trials are simple to carry out and demand only necessary laboratory apparatus and express high reactivity and specificity. They are, however, more expensive, even more so if the incubation time is reduced, and they take too long for same-day results.

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