Comprehensive bacteriological risk assessment of drinking water sources using multiplex PCR and real time PCR

Thesis submitted to the University of Calicut for the award of the degree of

DOCTOR OF PHIOLOSOPHY IN MICROBIOLOGY

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Under the guidance of
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CERTIFICATE

This is to certify that this thesis entitled **"Comprehensive bacteriological risk assessment of drinking water sources using multiplex PCR and real time PCR."** is a bonafide research work done by **Mrs. Ambili M,** under my supervision and guidance in the Department of Life Sciences, University of Calicut, for the award of the degree of Doctor of Philosophy in Microbiology, under the faculty of Science of the University of Calicut. I also certify that the same has not been submitted for any other degree diploma or associateship in any other University.

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DECLARATION

I, Ambili M, hereby declare that this thesis entitled **"Comprehensive bacteriological risk assessment of drinking water sources using multiplex PCR and real time PCR"** is being submitted to the University of Calicut in partial fulfillment of the requirement of the degree of Doctor of Philosophy in Microbiology under the faculty of Science. This thesis is the result of my work carried out in the Department of Life Sciences under the guidance and supervision of Dr.Denoj Sebastian, Assistant Professor in Microbiology, Department of Life Sciences, University of Calicut. This thesis or any part thereof has not been submitted for any other degree, diploma or any other similar title of any university.

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Ambili M

Dedicated to ……

All those who supported me in this endeavor

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LIST OF ACRONYMS USED

Acronyms	:	Expansion
AGE	:	Agarose gel electrophoresis
ATCC	:	American Type Culture Collection
CDC	:	Centre for Disease Control
CFU	:	Colony Forming Unit
CONS	:	Coagulase Negative Staphylococcus
CPCB	:	Central Pollution Control Board
DNA	:	Deoxy ribonucleic acid
dNTP	:	Deoxy nucleoside triphosphate
EHEC	:	Entero haemorrhagic *E. coli*
ETEC	:	Entero toxigenic *E. coli*
EPEC	:	Entero pathogenic *E. coli*
EIEC	:	Entero invasive *E. coli*
EAEC	:	Entero aggregative *E. coli*
EDTA	:	Ethylene diamine tetra acetic acid
EMB	:	Eosine Methylene Blue Agar
EtBr	:	Ethidium bromide
IMViC	:	IMViC: Indole, Methyl red, Voges-Proskauer, Citrate
FRET	:	Flourescence Resonance Energy Transfer
FISH	:	Flourescent Insitu Hybridisation
LB	:	Luria Bertani
MAC	:	Mycobacterium Avium Complex
MDG	:	Millennium Development Goals
MDR	:	Multi Drug Resistance
MPN	:	Most Probable Number
MR-VP	:	Methyl Red Vogues Proskauer
MSA	:	Mannitol Salt Agar

MTCC	:	Microbial type culture collection
NB	:	Nutrient Broth
NSF	:	Non Sorbitol Fermentor
PCR	:	Polymerase chain reaction
qPCR	:	Quantitative PCR
RNA	:	Ribonucleicacid
RPM	:	Rotation per minute
SAA	:	Starch Ampicillin Agar
SDS	:	Sodium Dodecyl Sulphate
TCBSA	:	Thiosulfate Citrate Bile Salt Sucrose Agar
TE	:	Tris EDTA
TSI	:	Triple Sugar Iron Agar
TVC	:	Total Viable Count
UV	:	UltraViolet
USEPA	:	United States environmental Protection Agency
VBNC	:	Viable But Non-Cultivable Bacteria
WHO	:	World Health Organisation
WSP	:	Water Safety Plans
YSA	:	Yersinia selective agar

CHAPTER 1

GENERAL INTRODUCTION

Water is the universal solvent to have a crucial role in all life forms and is used for drinking, recreational activities, and domestic purposes. It is, therefore, a vital resource to secure human well-being and health. There is no existence of life without water. Earth is the only known planet that has water to support life. Increasing population and economic development lead to increasing demand and severe water crises. India, a developing country, will face severe water scarcity problems in the future because of the lack of corrective measures (Hegde, 2012). WHO generates a specific strategy to maintain water quality and access safe drinking water and water for domestic purposes, creating and promoting socio-economic development (WHO, 2013).

About 75 % of the earth's surface is covered with water, 96.5 % in the ocean, which cannot drink 1.7 % in groundwater, glaciers, ice in Antarctica and Greenland, and other larger water bodies. About 0.001 % of water is present in the form of vapor clouds (Gleick, 1993). Only 2.5 % of the total water content on earth is fresh water, in which 98.8 % is present in ice and groundwater and about 0.3 % of total freshwater present in rivers, lakes, and the atmosphere. Physical bodies contain 0.003 % of water content. Earth has water content of 366 quintillion gallons, but only 0.007 % is potable. We need daily uptake of 2-5 litres of water for our normal functioning of the body. The human body contains 55-78 % of water. Potable water is used for human consumption.

Due to the high cost of drinking water, many industries use

treated surface water. However, it contains a high content of microbes, suspended solids, organic matter, etc. Potable water supply can come from many possible sources including public water supply well water and processed water. Humans are the major cause of water contamination. Many toxic substances are produced in the industries that are directly pumped to water bodies that will create many problems such as an increase in suspended solids, microbial growth, etc. that will cause serious health issues. According to the CDC report, more than 2 billion people, especially in developed countries, have no access to safe drinking water (Scallan, E. *et al.,* 2011(a); Scallan E. *et al*., 2011(b); Dusetty, P. *et al*., 2013). That will lead to a high rate of occurrence of waterborne diseases due to contaminated water consumption. Diarrhea is a major waterborne disease that occurs globally, along with gastroenteritis and infectious hepatitis. It results in the death of over 8 lakh children each year, the most common and the third major cause of infant mortality (Momtaz, H. *et al*., 2013).

Pathogens are found in the infected person's feces that subsequently come into contact with water sources, contributing to epidemics. Many are resistant to high pH, temperature, humidity, and disinfection, leading to survival in the aquatic environment (Ashbolt, 2004). *E. coli* is the major etiological agent of diarrhea and intestinal inhabitant of humans and other warm-blooded animals that easily enter the water source due to fecal contamination. USEPA published new guidelines for recreational water pathogen control in 2012, >126

CFU of *E. coli* is considered as non-potable (EPA Washington, 2012). Fecal contamination is measured primarily by the enumeration of indicator microorganisms, which may indicate that the sample has been exposed to or possess pathogenic microorganisms. The conventional culture methods are laborious, which cannot offer proper monitoring, and also some of these undergo a VBNC state, which cannot be detected in traditional culture methods (Juhna *et al*., 2007). By using rapid detection methods, these drawbacks can be eliminated. Several rapid detection methods are now available such as molecular methods, biosensor technologies, nanotechnology-based detection methods, electrochemical-based methods, etc. (Ramirez-Castillo *et al*., 2015).

Transmission pathways

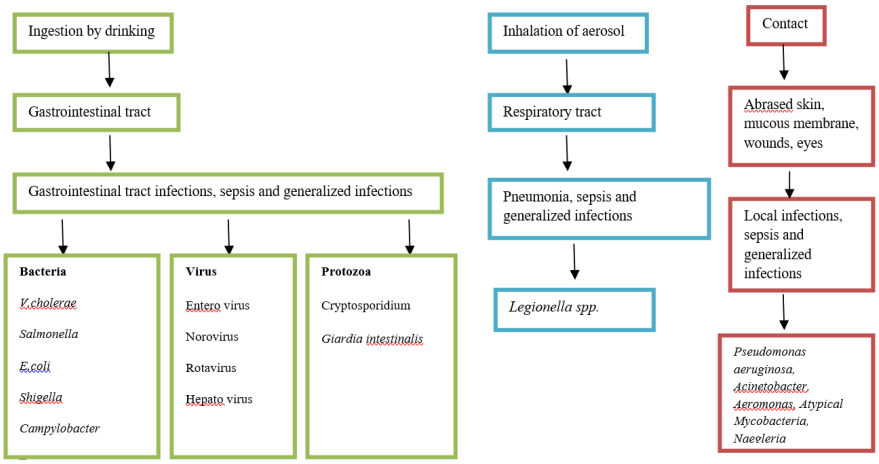


Figure 1.1. Transmission pathways Source: (WHO, 2004)

1.1. Waterborne pathogens and worldwide incidence

Waterborne pathogens are mainly three categories-bacteria, viruses and parasites, comprised of protozoa and helminths. WHO collated relevant waterborne pathogens (WHO, 2011) presented in Tables 1.1, 1.2, and 1.3.

The mode of transmission of **Enteric viruses** is through ingesting contaminated food and water. Table 1.1 shows some waterborne viruses categorized by WHO are summarized below.

Table 1.1. Overview of waterborne viruses

Pathogens	Disease	Relative infective dose	Route of transmission	Health significance
Adeno virus	Gastroenteritis, Respiratory infection	High	Ingestion, Inhalation	High
Astro virus	Gastroenteritis	High	Ingestion	High
Coxsackie virus	Hand foot and mouth disease	High	Ingestion	High
Echo virus	Gastroenteritis	High	Ingestion	High
Entero virus	Gastroenteritis	High	Ingestion, Inhalation	High
Hepatitis A and E	Hepatitis	High	Ingestion	High
Polio virus	Polio	High	Ingestion	High
Noro virus	Gastroenteritis	High	Ingestion	High
Rota virus	Gastroenteritis	High	Ingestion	High
Sapo virus	Gastroenteritis	High	Ingestion	High
Tora virus	Gastroenteritis	High	Ingestion	High

Waterborne bacterial diseases are the illness due to the consumption

of water that is contaminated with bacterial pathogens. Common bacterial diseases follow.

Table 1.2. Overview of waterborne bacteria

Pathogens	Disease	Relative infective dose	Route of transmission	Health significance
Campylobacter jejuni, Campylobacter coli	Gastroenteritis	Moderate	Ingestion	High
Pathogenic *E. coli*	Gastroenteritis	High	Ingestion	High
Salmonella typhi	Typhoid fever	High	Ingestion	High
Other *salmonellae*	Gastroenteritis	High	Ingestion	High
Shigella spp.	Dysentery	Moderate	Ingestion	High
Vibrio cholerae	Cholera	High	Ingestion	High
Yersinia enterocolitica	Enterocolitis	High	Ingestion	High
Pseudomonas aeruginosa	Gastrointestinal infections	High	Ingestion	Moderate
Francisella tularensis	Tularemia	High	Ingestion	High
Aeromonas spp.	Gastroenteritis	High	Ingestion	Moderate

The relative infective dose is required to cause infection in 50 percent of healthy adult volunteers (WHO, 2011).

Waterborne parasitic illness ranges from mild to chronic conditions that may lead to death in many conditions. The table 1.3 shows existing and emerging waterborne parasitic infections.

Table 1.3. Overview of waterborne parasites

Pathogens	Disease	Relative infective dose	Route of transmission	Health significance
Cryptosporidium parvum	Cryptosporidiosis	High	Ingestion	High
Enterocytozoon bieneusi	Microsporidiosis	High	Ingestion	High
Encephalitozoon intestinalis				
Encephalitozoon spp.				
Vittaformacorneae				
Nosema spp				
Cyclospora cayetenensis	Cyclosporiasis	High	Ingestion	High
Blastocystis hominis	Blastocystosis	High	Ingestion	High
Toxoplasma gondii	Toxoplasmosis	High	Ingestion	High
Taenia solium larva	Cysticercosis	High	Ingestion	High
Cysticercuscellulosae				
Clonorchia sinensis	Trematodiasis	High	Ingestion	High
Fasciola spp.				
Opisthorchis spp.				
Paragonimus spp.				
Echinostoma spp.				
Fasciolopsisbuski				
Giardia lamblia	Giardiasis	High	Ingestion	High
Naegleria fowleri	Naegleriasis	High	Ingestion	High
Entamoeba histolytica	Amoebiasis	High	Ingestion	High

Mainly waterborne diseases are reported in low-income countries with no access to safe drinking water. Furthermore, in developing countries, including India, safe drinking water is also a

significant problem forcing people to use unsafe water (Mukhopadhyay, C. *et al*., 2012; Chauhan, A. *et al*., 2015).

- Water-Washed Diseases: Diseases due to the lack of personal hygiene. Eg: Scabies, Conjunctivitis, Skin infections, and ulcers, etc. (Gerba & Pepper, 2019)

- Water-Based Diseases: Diseases caused by parasites, part of their life cycle spent in the water. Eg: Schistosomiasis, Dracunculiasis, etc (Gerba & Pepper, 2019)

- Water-Related Diseases: Diseases are not directly related to water quality. These are the diseases spread through waterborne vectors. Eg: Dengue, Filariasis, Malaria, Trypanosomiasis, and Yellow fever.

1.2. Microbiological aspects of drinking water

Developing countries have inadequate access to safe drinking water, so waterborne diseases are widespread in these regions. Mortality due to waterborne diseases is 5 million, including 1.5 million children annually (Fenwick, 2006). The major microbial risks associated with waterborne diseases are human or animal fecal contamination and wastewater discharges (George, I. *et al*., 2001). The Human waste is a major contributor to poor water quality (Karkey *et al*., 2016). Pre-screening of water helps detect the risk of pathogens and thereby helps to reduce the chance of outbreaks.

1.2.1. Fecal indicators in drinking water

Figure 1.2. The relationship between coliforms

1.2.1.1. Coliforms: Gram-negative non-spore-forming facultative anaerobic oxidase-negative lactose fermenting bacilli and produce acid and gas within 48 hours of incubation at 36±2 °C. And these are not ideal fecal pollution indicators.

1.2.1.2. Thermotolerant coliforms: The coliform group can ferment lactose by producing acid and gas at 44.5± 0.2 °C within 24±2 hours, also known as fecal coliforms.

Escherichia coli (E. coli): These are ideal fecal indicators and thermophilic coliforms, producing indole from tryptophan and β-glucuronidase.

1.2.1.3. Fecal streptococci (Enterococci): These are Gram-positive, catalase-negative cocci and can be differentiated from other Streptococcal groups by growing at 45 °C, pH 9.6, and 6.5 % NaCl. From the Streptococcal groups, *S. bovis* and *S. equinus* are known as fecal streptococci. Fecal Streptococci have many advantages over coliforms, including the resistance to environmental stress conditions

and can persist for a longer time (Gerba & Pepper, 2019)

1.2.1.4. Sulfite-reducing clostridia (SRC): Gram-positive, obligate anaerobic bacilli with spores, non-motile, can reduce sulfite to H_2S. *Cl. perfringens* is the most important species in this category.

1.2.1.5. *Clostridium perfringens*: Gram-positive strict anaerobic bacilli are closely associated with human fecal contamination and specific to sewage pollution, and they can ferment various sugars with the production of gas, resulting in stormy fermentation (Stelma, 2018).

1.2.1.6. Bifidobacteria: These are obligate anaerobic gram-positive, non-acid-fast, non-motile, and non-sporing bacilli with a highly pleomorphic nature. They are lactose fermenting catalase-negative organisms (except *B. asteroids, B. indicium*, and *B. coryneform*) and are considered as one of the major fecal indicators of recent fecal contamination. Bifidobacterial species, *B. dentium* and *B. adolescentis* are considered the strictly human origin and used for human fecal contamination detection in water (King, E.L. *et al* ., 2007; Nebra, Y. *et al*., 2003).

1.2.1.7. Bacteriophages (phages): Bacteriophages are major fecal indicators, mainly most somatic coliphages. The target is *E. coli* and infects through cell wall receptors. F-specific RNA coliphages infect *E. coli* through sex pili and phages infecting *Bacteroides fragilis* (Gerba & Pepper, 2019).

1.2.1.8. Coli phages: Somatic coliphages attack *E. coli* strains

Table 1.4. Bacteriological Quality of Drinking water

Sl No.	Organisms	Requirements
1	i) Water intended for drinking:	
	a) *E. coli* or thermotolerant coliform	Shall not be detectable in any 100 ml sample
2	ii) Treated water entering the distribution system:	
	a) *E. coli* or thermotolerant coliform	Shall not be detectable in any 100 ml sample
	b) Total coliform bacteria	Shall not be detectable in any 100 ml sample
3	iii) Treated water in the distribution system:	
	a) *E. coli* or thermotolerant coliform	Shall not be detectable in any 100 ml sample
	b)Total coliform bacteria	Shall not be detectable in any 100 ml sample

Source: Indian standard Drinking Water- Specification (Second Revision-2012)

1.3. Overview of waterborne bacterial pathogens

Infectious Dose

Infection usually occurs only when a sufficient number of organisms have been ingested, and the infectious dose of every organism varies. In the case of *Shigella spp*., ten organisms whereas *Campylobacter spp*., 100 cells. The infectious dose of *Vibrio cholerae*

is in the range of 10^6 in normal conditions, but when antacids increase the gastric pH, the infectious dose becomes 10^4. A high infectious dose means the waterborne transmission of these ofrganisms was difficult because of the low concentration in the natural environment (Madigan, M.T, *et al* ., 2000).

1.3.1. Vibrio cholerae

These are the etiological agent of severe, watery diarrhea with a characteristic rice water stool named cholera by cholera toxin. About 206 serogroups are reported based on the structure of lipopolysaccharide, in which O1 and O139 are the main etiological agents. Non-O1 and non O139 are found in aquatic systems (Bhattacharya *et al*., 1993; Kaper, J.B, *et al*., 1995; Moore, S *et al*., 2014). O1 can be classified as Classical and El Tor biotypes. The Classical and El Tor biotypes are differentiated using biochemical and virological characteristics (Shida, K *et al*., 1973; Cho, Y J *et al*., 2010). The infective dose is about 10^2-10^4 bacteria are adequate to cause infection.

1.3.2. Campylobacter spp.

There are several species of *Campylobacter* species that were identified. Among these, *C. jejuni* is the most important species; apart from this other species *C. coli, C. laridis* and *C. fetus* have also been found. *Campylobacter* is gram-negative curved bacilli with polar flagellum, culturally microaerophilic and capnophilic organisms. An infectious dose of fewer than 1000 cells is required. It can produce

campylobacteriosis by consuming contaminated water, which is generally a self-limiting infection that rarely manifests complications such as Reiter's syndrome, Guillen-Barre syndrome, etc. The prevalence of organisms is related to high rainfall (Bridle, 2013). Animal reservoirs, mainly poultry, are also reported (Medema, G J *et al*., 1996). Outbreaks are largely due to the result of inadequate water treatment (Levin, 2007). These are sensitive to disinfectants, and survival capability in the aquatic environment is for many months (Lund, 1996; Szewzyk, U *et al*., 2000).

1.3.3. Shigella spp.

These are gram-negative, non-motile non-sporing bacilli, which are facultative anaerobes. There, are 4 species, namely, *S. dysentriae, S. flexneri, S. boydii* and *S. sonnei*. From this *S. dysentriae* is more pathogenic can cause ulceration and bloody diarrhea called bacillary dysentery. *S. sonnei* can cause self-limiting diarrhea (DuPont, H L *et al*., 1989). The infectious dose is significantly less than 100 cells. There are more than 2 million cases are reported annually with a high mortality rate.

1.3.4. Legionella pneumophila and *Other Mycobacterium spp*.

L. pneumophila is prevalent in aquatic environments, air conditioners, and plumbing infrastructures. Moreover, *Legionella* and *Mycobacterium avium* complex (MAC) can grow and proliferate in

water, cooling towers, and hot water supplies. The etiological agent of Legionnaires disease is *L. pneumophila* through the hospital and residential water supplies (Fliermans, 1996; Rowbotham, 1980). Many studies suggest *M. avium* complex is also responsible for waterborne infections (Collins, C H *et al*.,1984; Von Reyn, C F *et al*., 1994).

1.3.5. Aeromonas spp.

Many studies have shown that *Aeromonas* may be the causative agent of diarrheal illness. (Albert *et al*., 2000). The virulence of *Aeromonads* is strain-specific. *Aeromonas spp*. are associated with natural water sources; most available strains represented the natural inhabitant of the organism (Leclerc, H *et al*., 2002). Burke *et al.,* (1984) observed that *Aeromonas spp.* can cause gastroenteritis closely related with organism present in the water sample. The epidemiological relation between *Aeromonas* isolated from humans and water samples has been studied using typing by Havelaar. This study reveals no correlation between *Aeromonas* isolated from patients and water samples (Havelaar, A H *et al*., 1990). Even though the organism is frequently isolated, no *Aeromonas* diarrhoeal outbreaks have been reported (Schubert, 1991).

1.3.6. Yersinia enterocolitica

Yersinia enterocolitica is responsible for waterborne gastrointestinal outbreaks and is associated with well water samples.

Studies showed that the environmental strains of *Yersinia* should be differentiated from the serotypes, O:3, O:9, O:5, O:27, and O:8, responsible for many gastrointestinal infections globally (Schiemann, 1990). They are considered environmental strains because they are prevalent in the aquatic environment, such as non-pathogenic. *Y. enterocolitica*, *Y. bercovieri*, *Y. frederiksenii, Y. intermedia,* and *Y. kristensenii*, *Y. mollaretii*, *Y. rohdei,* and *Y. aldovae* (Langeland, 1983; Aleksic & Bockemuhl, 1988).

1.3.7. E. coli

E. coli is the normal flora of the GI tract of humans and other warm-blooded animals. *E. coli* is used as a fecal indicator in distilled water surveillance. Human pathogenic *E. coli* are diarrhoeagenic type, mainly Enterohaemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC) and Enteroaggregative *E. coli* (EAEC) (Mead & Griffin, 1998). *E. coli* O157: H7 belongs to the EHEC group. It was detected as a potent human pathogen in 1982 based on two outbreaks of bloody colitis. It is one of the most frequently reported waterborne pathogens. Infants and older adults are more susceptible to *E. coli* infections (Doyle, 1990).

1.3.8. Salmonella enterica

These are gram-negative, motile and most of them are responsible for H_2S production from carbohydrate fermentation. There are two main species, namely, *S. enterica* and *S. bongori*, with 2000

subspecies. *S. typhi* is a human pathogen with high host specificity. *S. enterica* causes self-limiting diarrhea with the onset of 6-72 hours after consuming contaminated food/water lasting for 3-5 days. Typhoid species are most responsible for waterborne diseases, with a 1-14 days incubation period causing fatal typhoid fever. Several outbreaks were reported with *S. enterica* (Lynch, M F *et al*., 2009; Kovačić, A *et al*., 2017). Non-typhoidal species rarely cause waterborne outbreaks. These organisms are sensitive to chlorine disinfection.

1.3.9. Pseudomonas aeruginosa

It is a gram-negative, motile, and non-sporing bacteria that can adapt to various environmental conditions such as water, soil, clinical settings, etc. (Pellett, S *et al*., 1983; Cavalca *et al*., 2000; Wolfgang *et al*., 2003; Kimata, N *et al*., 2004). These are found in many drinking water sources (Hunter, 1993; Trautmann *et al*, 2001; Naze *et al*., 2010). These are responsible for many outbreaks related to water consumption and high morbidity and mortality rates with immune-compromised patients.

1.4. Monitoring of waterborne pathogens- From culture to genomics

Routine waterborne pathogen monitoring is vital in the case of assessing safe drinking water. Investigative monitoring helps select the proper microbial barriers to remove the pathogens and detect outbreak

sources, thereby preventing the reoccurrence of outbreaks. This type of monitoring is necessary to obtain sufficient information about the quality of water, which aids in determining the pathogen's qualitative and quantitative features (Bridle, 2013).

Operational monitoring attempts to give convenient signs of presenting any actualized drinking water treatment measure, empowering the chance to make the right move to remediate any possible issues. The critical factor is rapid estimations, which deliver the information in an ideal time for action to be made. As per WHO, pathogen monitoring is restricted for operational purposes as existing methods are time-consuming and laborious. Surveillance monitoring gives data to evaluate the proper working of Water Safety Plans (WSPs) and provides effective administration and management of water resources to improve water supplies (Bridle, 2013).

Escherichia coli and other coliforms have been suggested as indicator organisms for routine drinking water quality monitoring. The presence of fecal indicators indicates the possible presence of other pathogens (Fatemeh *et al*., 2014). The issue with existing strategies is that the utilization of fecal indicators is not constantly corresponded with the presence of pathogens. Furthermore, the culture-based conventional methods are simple, easy, and tedious (Kong, R Y *et al*., 2002).

Waterborne pathogens are grouped into bacteria, cyanobacteria, protozoa, viruses, and helminths. There is no general technique to

collect, recognize, and distinguish all pathogenic microorganisms from a water test. Alongside the utilization of indicator organisms that might relate well with pathogen levels, the sample collection impacts the ability to identify the microorganism. According to WHO, water quality monitoring focuses mainly on three bacterial groups: coliform, thermotolerant coliform, and *E. coli* (WHO, 2011).

E. coli is considered a fecal indicator. Recognition of indicator organisms is probably an ideal approach to assess the effectiveness of water disinfection techniques. Regarding their significance, the most significant indicator organisms include *E. coli*, coliforms, and other thermotolerant coliforms.These organisms' presence in the water indicates insufficient disinfection and recent and frequent human and warm-blooded animals fecal contamination. Thermotolerant coliforms, aside from *E. coli*, can enter the drinking water through industrial effluents and soil disintegration. Conventional culture-based techniques have restrictions, such as long incubation period, cross-contamination, inability to detect “viable but non-cultivable bacteria” (VBNC), and adverse environmental conditions stress. PCR has been suggested as a rapid, sensitive, and specific technique for detecting coliforms in drinking water. PCR-based methods have many advantages, such as specificity, sensitivity, and rapidity of the tests, reducing workload. In these techniques, the DNA of the target organism is amplified using specific oligonucleotide primers. For example, the *lacZ* gene (β-galactosidase) is used to recognize total coliforms, and *uidA* (β-glucuronidase) for *E. coli* is used. Furthermore,

dctA, dcuB, frdA, dcuS, and *dcuR* are specifically used for *E. coli* in the water sample (Fatemeh *et al*., 2014).

Research objectives

Even though the water quality analysis techniques have been improved in recent years, most improvements have been achieved by using rapid, sensitive, and specific methods, mainly nucleic acid-based methods. Nonetheless, it is possible to further improve by the co-detection and enumeration of multiple waterborne pathogens. This work explores the possibility of PCR-based methods in water quality monitoring, mainly multiplex PCR and qPCR. The present work deals with the following objectives;

- Development of multiplex PCR for the rapid detection of waterborne pathogens.
- Construction of standard curve using a known concentration of reference strains for the absolute quantification method and thereby detect pathogen load in the unknown samples.
- Cost-effective survey for the quality of drinking water using PCR-based and conventional culture methods.

The remainder of this thesis is organized into five chapters: Chapter 2 describes the literature review, Chapter 3 discusses the preliminary screening for the detection of fecal coliforms and prevalence of other waterborne pathogens in drinking water in the Malabar region of Kerala, Chapter 4 introduces a novel method for the

co-detection and enumeration of several waterborne pathogens using multiplex PCR and real-time PCR assays, Chapter 5 outlines the experimental application of these developed molecular methods to the water samples; Chapter 6 concludes this work with the summary of results.

CHAPTER 2

LITERATURE REVIEW

Consequently, inadequate supplies of safe water, disposal methods, water shortages, and insufficient hygiene create optimum conditions under which oral-fecal diseases continue to flourish. According to WHO, when a community strengthens its water supply, sanitation, and hygiene, health will improve and shorten diarrheal diseases by 26 %. In a global scenario, almost 1 billion people have no access to safe drinking water, and about 260 million people suffer from water-borne diseases (WHO and UNICEF, 2014).

For this reason, poor water quality is considered a significant threat to humans. Water-borne diseases are transmitted through the water, which is contaminated with bacteria, viruses, protozoa, and intestinal parasites. Many water-borne diseases are characterized by diarrhea followed by dehydration and can result in death in severe cases. Water-borne diseases should be considered a severe problem because they rapidly affect large sections of the population, leading to a high disease burden that will significantly impact the country's economy.

Microbial causes constitute a significant problem regarding water-borne infections. Primary etiological agents include bacteria, viruses, parasites, and protozoa found in the fecal matter of infected persons and transmitted through the fecal-oral route. In tropical regions, more prevalent infections include cholera, dysentery, hepatitis, and typhoid (THY, 1992).

The major sources of these pathogens include point sources and nonpoint sources. Point sources are mainly sewage discharges and they

can be easily managed by treatment. Nonpoint sources, including urban water runoff, wildlife, and agriculture waters, are wide and very threatening. The major etiology includes *Vibrio cholerae*, *E. coli*, *Shigella spp., Campylobacter spp.*, *Salmonella spp., Yersinia spp.*, enterovirus, adenovirus, hepatitis A and E virus, coxsackievirus, norovirus, echovirus, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Schistosoma*, *Echinococcus granulosus, Dracunculus medinensis*, *Giardia lamblia,* etc. (Lipp *et al.*, 2001; Noble & Fuhrman, 2001; Choi & Jiang, 2005; Rose *et al.*, 2006; Jiang *et al.*, 2007; Nenonen *et al.*, 2008; Symonds *et al.*, 2009).

Microbial water contamination is a significant public health problem leading to large-scale water-borne diseases and outbreaks (Szewzyk *et al.*, 2000). Water-borne diseases are frequently reported even in developed countries that maintain strict water quality standards. US Centre for Disease Control, 2013 confirmed about 33 and 81 outbreaks among 1040 drinking water-related and 1326 recreational water-related diseases, respectively (CDC, 2013; Hlavsa *et al.*, 2014). In 2017, 71 % of the global population used an adequately managed drinking-water service free from contamination. There is even a lack of essential drinking water services for 785 million people, including one hundred and forty-four million dependent on surface water. Globally, at least 2 billion people use fecally contaminated drinking water (U. WHO, 2017). Water-borne diseases are more prevalent in low and middle-income countries like Africa and Southeast Asia (Johansson *et al.*, 2012).

In India, annual cases of 37.7 million people affected with water-borne diseases, and 1.5 million children die of diarrhea, leading to an annual economic burden of $ 600 million. About 10,738 deaths have occurred due to water-borne diseases since 2017, in which acute bacterial diarrheal infections become the most critical illness, followed by viral hepatitis and cholera. The state-wise picture shows Uttar Pradesh recorded the highest number of death, followed by West Bengal, Assam, Odisha, and Madhya Pradesh. Open defecation and lack of proper sewage disposal mechanisms increase the chances of infection (Central Bureau of Health Intelligence, 2018).

States with high diarrheal mortality in 2017, India

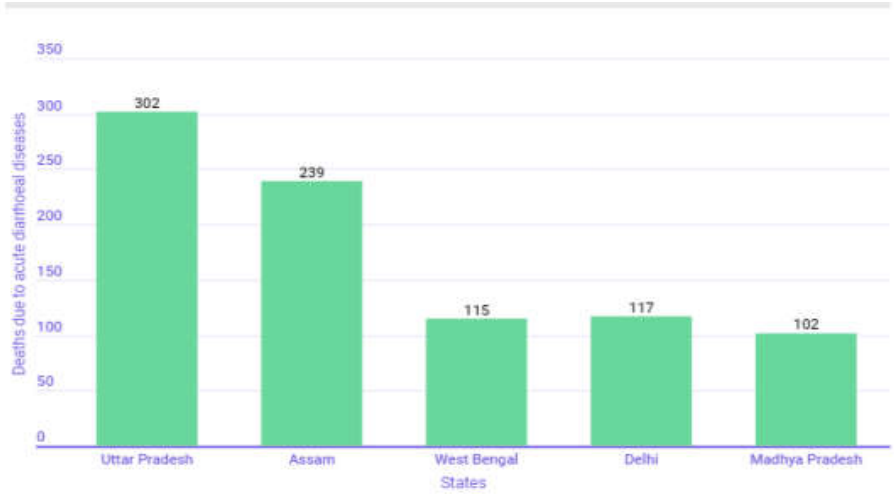


Figure 2.1. Source: CBHI (2018) National Health Profile 2018, 13th Issue

The essential factors for human health and well-being outcomes include access to safe drinking water. According to the World Health Organization, the principal determinant of health is high-quality

drinking water -WHO 2010. In developing countries, including India, 80 % of all diseases are linked directly or indirectly to contaminated water, so safe drinking water is vital for human development (Sanitation, 2010; WHO, 2011).

Major water-borne microbial pathogens include bacteria, viruses, and protozoa (Girones *et al.*, 2010). In most water-borne outbreaks globally, pathogenic bacteria have been described as the major etiological agent (WHO, 2011). Approximately 165 million cases of bacterial diarrhoeal diseases worldwide are bacillary dysentery by *Shigella spp*. Of those, 163 million are in developing and 1.5 million in developed countries, with an approximate annual death rate of 1.1 million (Sharma, A *et al.*, 2010). Most *Arcobacter spp.* was detected from river water using multiplex PCR found in river water. 533 isolated from various sources of environmental water, including surface and groundwater. Fecal indicators and meat, especially from poultry, pork, and beef, have been associated with the presence of these organisms (Fong *et al.*, 2007; Collado *et al.*, 2008). Some *Arcobacter* species, such as *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*, have been involved in animal and human diarrhea cases, indicating a fecal-oral transmission route to humans and animals. (Gonzalez *et al.*, 2007). On the other hand, *Helicobacter pylori,* found in surface water and wastewater, has been involved in gastric, peptic, and duodenal ulcers (Linke *et al.*, 2010).

Biofilms in drinking water distribution systems have been identified as potential reservoirs of *H. pylori* and try to culture these cells from ineffective water samples (Percival & Thomas, 2009; Linke

et al., 2010). Very few quantitative studies have been reported due to the fastidious nature, the lack of standard culture methods for environmental samples, and the inability to survive in an infectious state in the environment (Percival & Thomas, 2009). *Legionella pneumophila* is a common bacteria found in natural aquatic ecosystems that can survive in human-controlled water systems such as air conditioning and plumbing (Steinert *et al.*, 2002). *Vibrio vulnificus*, an opportunistic human pathogen that causes gastroenteritis, serious necrotizing soft tissue infections, and primary septicemia, is also found in fish, shellfish, water, and wastewater. Generally, infection is related to the intake of infected fish and water. (Harwood *et al.*, 2004). Also, the presence of *Salmonella, Shigella, E. coli,* and *Klebsiella* in water has been described as a severe threat to human health and etiological agents of many diseases (Leclerc *et al.*, 2001).

The most common etiological agents of human enteric fever and bacterial gastroenteritis are *Salmonellae*, water- and shellfish-transmitted human disease. It is frequently isolated from marine water, where it can remain viable for several hours (Malorny *et al.*, 2008). *Salmonella* contamination has been identified in recreational surface water, drinking water, and irrigation sources, demonstrating the potential risk of contaminated water (Gannon *et al.*, 2004). Typhoid is caused by *S. enterica*. Serotype *typhi* is a severe public health hazard in developing countries, and the emergence of various drug-resistant *S. typhi* exacerbates the global typhoid fever epidemic. *Salmonella* is prevalent in natural water resources due to contamination from animal husbandry activities and untreated waste discharge (Jenkins *et al.*, 2008). Low numbers of S*almonella* can pose a public health risk in

food, recreational, surface, and potable water supplies as their infectious dose may be as low as 15-100 CFU (Cobbold *et al.*, 2006).

Significant human pathogens known as the leading cause of bacillary dysentery are *Shigella* and enteroinvasive *Escherichia coli* (EIEC) (Szakal *et al.*, 2003). The infectious dose of *Shigella* is very low (10^1-10^4 organisms), while a larger infectious dose is needed for EIEC strains (between 10^6 and 10^{10} organisms). Invasion plasmid antigen, which is regarded as a key virulence protein and is utilized as a molecular marker in the case of Polymerase Chain Reaction detection, is found in both *Shigella species* and EIEC. Both are transmitted through direct contact from human-to-human interaction or through contaminated food and water. Clinical characteristics of bacillary dysentery caused by EIEC that mimic shigellosis include fever, abdominal cramps, malaise, toxemia, and watery diarrhea. The *E. coli* -serotype O157: H7, an emerging fecal pathogen isolated from water, has been involved in food and water-borne diseases. (Bavaro, 2009).

Klebsiella is the most prevalent cause of nosocomial infections. The mammalian gastrointestinal tract and environmental sources such as soil, surface water, and plants serve as non-clinical habitats. Environmental isolates have been described as indistinguishable from human clinical isolates in biochemical reactions and virulence. Although *Klebsiella*'s medical relevance is isolated from the natural environment, such habitats are thought to be possible reservoirs for the growth and spread of these bacteria that can colonize animals and humans. *K. pneumoniae* and *K. oxytoca*, the most clinically significant

opportunistic pathogens among the five *Klebsiella* species investigated, have been verified to be present in water in community-acquired pyogenic liver abscess and bacterial meningitis cases in adults (Field *et al.*, 2003).

Large numbers of *Escherichia coli* bacteria exist as human intestinal origin. Diarrheagenic strains can cause acute diarrhea. Based on the virulence these strains have been classified as, enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). *E. coli* O157: H7 (an EHEC) is one of the major diarrheagenic strains. The mode of transmission of these bacteria is ingestion, and it can lead to symptoms ranging from mild to highly bloody diarrhea, and it can lead to the hemolytic uremic syndrome in about 2-7 percent of cases. This latter illness, which is even more likely to occur in children under the age of 5, maybe fatal. The principal zoonotic source of pathogenic *E. coli* is EHEC, which infects cattle, sheep, goats, chickens, and pigs to a lesser degree. While EPEC strains may be less common than *Campylobacter* infection, the symptoms can be far more severe. Moreover, the infectious dose is low at less than 100 organisms. In 2000, 7 deaths and 2300 illnesses were caused by an outbreak in Walkerton, Canada. The largest-ever Vero cytotoxigenic *E. coli* outbreak occurred in Europe in 2011, with 3929 diseases and 47 deaths. This outbreak was attributed to the consumption of sprouts from infected seeds that may have been

contaminated by low-quality water for irrigation purposes (Field *et al.*, 2003).

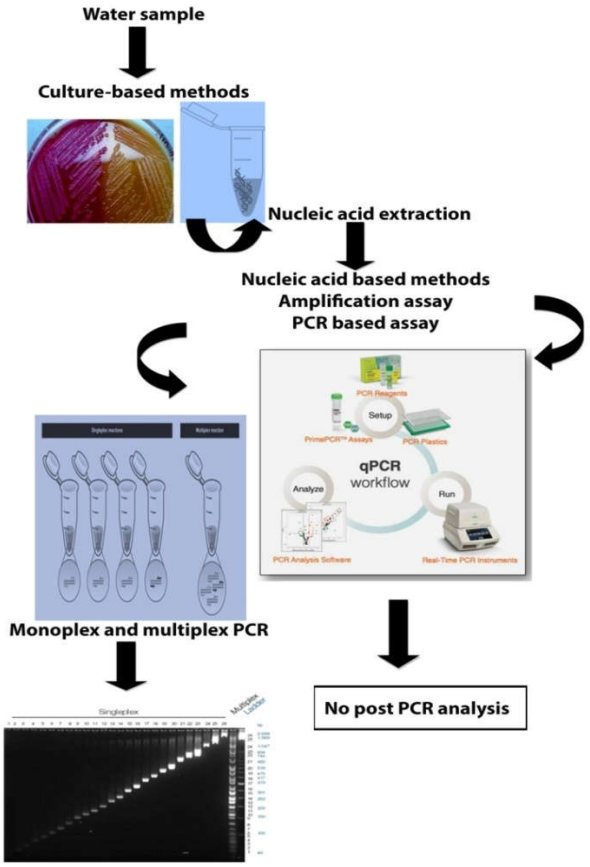


Figure 2.2. Schematic representation of water-borne pathogen detection-culture to genomics.

2.1. Conventional microbiological methods for water quality analysis

Bacteriological enumeration is used to detect pollution, detect specific target groups, and ensure conformity of water quality guidelines. The microbiological analysis includes methods to estimate the number of microorganisms as well as the type. This process is used to detect the quality of water. The common feature for routine analysis includes screening indicator organisms and a relationship between indicators and the possible presence of pathogens. Conventional culture methods MPN, membrane filtration methods, and standard plate count to detect CFU/ml.

2.1.1. Most Probable Number

The MPN test is a statistical test that estimates the number of fecal coliforms in a water sample based on the species' degree of lactose fermentation in the sample. A series of phenol red lactose broth tubes are inoculated in this test with measured quantities of water to determine if the water contains any lactose-fermenting bacteria that generate the gas.

It is considered that coliforms are present in the water sample if fermentation and gas generation occurs after incubation. The "most probable number" (MPN) of coliforms is statistically calculated using a standardized chart by counting the number of positive tubes at each dilution.

2.1.2. Quantitative analysis through total viable count

To isolate total viable bacteria, 0.1 ml of pre-enriched water samples were spread onto nutrient agar using the spread plate technique. The plates were incubated for 24 hours at 37 °C. All of the isolates were identified using both primary and secondary identification methods (Cappuccino & Sherman, 1996).

2.1.3. Membrane filtration

Membrane filtration can be used for the secondary concentration of bacteria and parasites in drinking water and other low turbidity water. Samples are filtered via flat, thin-sheet membranes during membrane filtration. These membranes are mostly polymeric with bacterial pore sizes of 0.22 or 0.45 μm and parasite sizes of <2 μm, and any content greater than the pore size is deposited on the filter. The filter is processed for bacteria according to standard methods for detecting bacterial pathogens in water, either put on a selective medium for quantitative or qualitative detection in an enrichment broth (Bridle, 2013). Direct microscopic analysis can then be carried out on parasites that are filtered onto membranes. But conventional membrane filtration filters have some issues, including a tortuous pore path, low pore density, overlapping pores, and a high coefficient of variation, all of which lead to relatively low sample throughput and cell recovery rate (Lee *et al.*, 2010).

2.1.4. World scenario on the bacteriological quality of drinking water

Modrzewska, Barbara D *et al.,* (2019) analyzed bacteriological and mycological parameters for detecting the microbial quality of water samples. They found that *E. coli*, other coliforms, and pathogenic fungi are the primary contaminants. Their study suggests that these fountains are the major hidden sources for the epidemiological problem. A study from boreholes, the major drinking water source in Thulamela Municipality, South Africa, revealed a high microbial load of fecal indicators in the summer than in the monsoon season (Enitan-Folami *et al.*, 2019). A study by Khan *et al.,* (2019) aimed to detect the microbial quality of public water supply and borehole water in Karachi, Pakistan. In his findings, about 96 % of the samples showed the presence of total coliforms, and the total viable count was >200 CFU/ml.

Oluyege *et al.,* (2019) studied the microbial quality of drinking water from Nigeria, of which a total of 272 *E. coli* was identified, and 150 of these isolates were non-sorbitol fermenters (NSF) and which is the feature of *E. coli* O157, and some of them showed high plasmid-mediated antibiotic resistance (Olowe *et al.*, 2019). In Mali, in a study conducted by Toure *et al.,* (2019) the fecal coliform count was higher than the permissible limit in the samples. Valsangiacomo *et al.,* (2019) conducted a comparative study on water quality before and after floods in Khyber Pakhtunkhwa, Pakistan, showing that post-flood well cleaning campaign successfully minimized fecal contamination.

Unfortunately, in the following months, the fecal coliform count was higher in those areas due to human activities.

In a study by Addo *et al.,* (2019) conducted in Sachet water, Ghanaian University found that one-half of the samples were highly contaminated with both total and fecal coliforms and the mean total coliform count /100ml were in the range of 9.15×10^5- 2.35×10^6 and fecal coliform counts of 2.3×10^5-4.15×10^5 respectively, along with *E. coli* count of 4.0×10^4. According to Ciftci *et al.,* (2019), the bacteriological quality of Sapanca lake, Turkey was inferior with $71\pm3.1\times10^4$ CFU/ml in summer. The potability analysis of tube wells in the Kushite district of Bangladesh showed 56.25 % of total samples were highly contaminated with FC and 68.75 % of TC (Rahman & Rahaman, 2018). Kayambe *et al.,* (2018) revealed the fecal contamination of the Kokolo canal and shallow wells of Congo with *E. coli* values 18.6×10^5 and 4.9×10^5 CFU/100ml respectively in the wet season.

A study from Nigeria showed that a high-level bacterial contamination of well water with TVC from 0.86×10^4CFU/ml to 3.04×10^4 CFU/ml and total coliform ranged from 0.24×10^2 CFU/ml to 1.84×10^2 CFU/ml. The prevalence of bacterial species showed *Staphylococcus aureus* was the highest (53.33%) followed by *E. coli*, *Pseudomonas spp*, *Proteus* species, *Salmonella* species, *Enterobacter* species, *Klebsiella* species, and *Enterococcus* species, respectively (Agwaranze *et al.*, 2017).

Many studies showed that drinking water in Pakistan revealed a high level of microbial contamination (Ahmed *et al.*, 2014; Abbas *et al.*, 2015), and also in South Africa, drinking water quality is very poor (Mulamattathil *et al.*, 2015; Palamuleni & Akoth, 2015). In 2016 Thani Suleiman Thani *et al.,* (2016) studied the persistence of *E. coli* in well water and borehole water in Mombasa coastal areas. The findings defined that the well water contaminated with 39.7 % of coliforms. They tested the incidence of *E. coli* by Eijkman test and observed that 60.3 % of the samples contained *E. coli*. The detection of the *E. coli* strains is simple but in the case of pathogenic strains, the conventional methods cannot help detect viable but nonculturable *E. coli.*

2.1.5. Indian scenario on the bacteriological quality of drinking water

Pankaj Kumar *et al.,* (2019) evaluated the bacteriological content of the bottled and bubble top packaged drinking water to determine the packaged drinking water sold in and around Kolkata and public water supply from Kolkata corporation, and all the samples were in excellent categories free from coliforms. The Gangetic delta analysis showed a high distribution of coliforms in the rainy season than in winter (Saha *et al.*, 2019). Mula Mutha river is related to many drinking water sources in Pune, and a study on these sites in 3 different seasons showed the highest thermotolerant fecal coliforms during monsoon at all eight sampling sites (Dhawde *et al.*, 2018). According to Joseph N *et al.,* (2018) bacteriological analysis of bottled drinking water marketed in major transit locations in Mangalore, Karnataka, in

62.5 % of the samples, the total coliform count was within an acceptable range. Lal B *et al.,* (2018) conducted a microbiological contamination survey in dental unit water systems in Chandigarh, with 97.7 % of total water supplies exceeding 100 CFU/100ml. The most prevalent isolates were *Acinetobacter, P. aeruginosa*, and *Sphingomonas paucimobilis.*

Open defecation is a major threat in many of the areas of India and contaminates the water sources with coliforms. A study from Chikhli proved that tap water and river water were seriously fecally contaminated which may be due to the percolation of wastewater and sewage (Garode & Bhusari, 2017). A study from many schools in Amritsar revealed that 39.8 % of tested samples were unfit for human consumption, so strict regular monitoring should be done because, in school, children are vulnerable to many diseases due to developing immune systems (Malhotra *et al.*, 2015). Antibiotic resistance of the fecal coliforms constitute a significant threat to the public, and there were many studies that showed the multiple antibiotic resistance of fecal coliforms isolated from water sources (Kucuk *et al.*, 2016).

2.2. Nucleic acid-based methods

Relatively small numbers of microorganisms accompanied by microflora are tough to detect. So, techniques of bacterial concentration have been developed to perform specific pathogen detection, thus preventing false-negative results (Fukushima *et al.*, 2007). Concentration is a crucial step in enhancing the sensitivity of

pathogen detection, particularly by molecular tools using minimal sample quantities (Aw & Rose, 2012). Concentration methods can be carried out under nonspecific strategies, including centrifugation, filtration dielectrophoresis, etc, and specific strategies with immune magnetic separation and flow cytometry (Dwivedi & Jaykus, 2011).

DNA extraction is the first step in PCR after the concentration and isolation of the bacteria. There are currently several DNA extraction procedures, and the methods should be selected based on the needs of researchers. In general, bacterial DNA extraction includes three sequential steps: the first is cell lysis, the second is the degradation of the protein fraction associated with DNA, and the last is DNA purification (Rodríguez & Rizo, 2011).

In PCR-based assays, specific DNA or RNA of the target pathogens, including toxin-producing genes, can be detected by hybridization with synthetic oligonucleotide sequences (Rompre *et al.*, 2002; Ramirez-Castillo *et al.*, 2015). The main advantages of nucleic acid methods include rapidity and time efficiency, and it is possible to perform without culture. Major nucleic acid-based methods include polymerase chain reaction and its variants (PCR), microarrays, pyrosequencing, and fluorescent in situ hybridization (FISH).

2.2.1 PCR Based methods

PCR is a molecular tool that enables oligonucleotide primers to amplify target DNA fragments in a sequence of replication cycles catalyzed by DNA polymerase (Taq polymerase) (Collado *et al.*, 2008; Clifford *et al.*, 2012). By targeting particular DNA sequences in a three-step cyclic process involving denaturation, annealing, and extension, PCR is the most widely used molecular tool for detecting water-borne pathogens (Maheux *et al.*, 2011; Mandal *et al.*, 2011). Denaturation is the unwinding of double-stranded DNA to single-stranded sequences, followed by primer annealing. And in extension, thermostable DNA polymerase carries out the polymerization in the presence of dNTPs and specific ions, so it helps to increase the specificity of target DNA detection in very low numbers in environmental samples (Khan & Edge, 2007; Maheux *et al.*, 2013). And the amplified products get detected by staining with ethidium bromide, a strong carcinogen, by agarose gel electrophoresis (Khan & Edge, 2007). This technique is high sensitive and specific for microbial identification and surveillance. It has been successfully applied in clinical and environmental samples to detect and identify pathogenic bacteria and the investigate outbreaks of food and water-borne diseases. In the environmental field, quantitative PCR (qPCR) is increasingly becoming known, as it is more sensitive in many cases than the bacterial culture or the viral plaque assay. However, unlike traditional culture-based techniques, molecular protocols do not differentiate between viable and non-viable species, so more knowledge is needed before

substituting molecular methods with conventional methods. There are also many advantages to molecular techniques for the specific detection and quantification of bacterial pathogens over traditional methods: high sensitivity and specificity, speed, ease of standardization, and automation. As with viruses, direct PCR amplification of these bacterial pathogens from water samples is challenging due to only a limited number of target bacteria in environmental sources. Therefore before performing a PCR, an enrichment step is generally required. Improved identification of Pathogenic *E. coli* by Immuno-capture PCR and the sensitive real-time PCR detection of *Salmonella* and *Campylobacter* have also been established, still these procedures are both monospecific and are laborious or very costly for routine use in laboratories for water testing. More recent developments have made the simultaneous identification of multiple microorganisms in a single assay. The use of multiplex polymerase chain reaction (m-PCR) helps to provide rapid, sensitive detection of specific pathogens in the aquatic environment (Girones *et al.*, 2010).

2.2.2 Multiplex PCR

Multiplex PCR is a variant type of PCR reaction in which more than two sets of primers are used in the same set of reaction tubes for which two or more targets get amplified, which helps to reduce time and effort. It was first described in 1988 (Chamberlain *et al.*, 1988), have greater advantages in the quantitative analysis (Zimmermann &Mannhalter, 1996), polymorphism studies (Shuber *et al.*, 1993),

mutation studies (Rithidech *et al.*, 1997) and gene deletion studies (Chamberlain *et al.*, 1988) and various significances in the case of the medical field for the detection of target pathogens (Heredia *et al.*, 1996; Hendolin *et al.*, 1997; Harris *et al.*, 1998; Markoulatos *et al.*, 2002). Because of the use of many primers, the optimization faces some difficulties, mainly the formation of primer dimmers, due to the high primer-to-template ratio or excess primers. If the primer-to-template ratio is meager that will affect the product yield. So primer design should be carried out very carefully with target sequence homology, GC content, length, and concentration (Brownie *et al.*, 1997) and for a successful multiplex PCR amplification, other factors such as PCR buffer concentration, $MgCl_2$ and dNTP concentrations, amount of template, Taq DNA polymerase and cycling temperatures are very important to get highly specific amplification products (Markoulatos *et al.*, 2002).

One of the most important indicators of water quality is *E. coli* so that many studies were conducted globally to identify the pathogenicity of *E. coli* in environmental water samples. The application of next-generation sequencing, which offers improved detection sensitivity with PCR methods, was studied by many researchers. Bo Li *et al.,* (2019) designed this type of assay for the direct detection of *E. coli, S. flexneri, S. enterica, C. jejuni, C. perfringens, L. pneumophila, L .monocytogenes* and *V. cholereae* (Li *et al.*, 2019). Bej *et al.,* (1991) and Godambe *et al.,* (2017), used a combination of conventional and molecular methods for the detection

of pathogenic *E. coli* strains. They worked on PCR with two primers (*uidA* and *uspA*). Although Godambe *et al.,* (2017) concluded that molecular-based techniques are the best way to identify pathogens.

Giowanella *et al.,* (2015) detected the pathogenic strains of *E. coli* by targeting specific virulence genes to detect diarrheagenic *E. coli*. Moreover, a study from the Philippines revealed that 58.22 % of MDR *E. coli* in agriculture irrigation water and also the prevalence of class I and class II integrons of the MDR *E. coli* can be detected by using multiplex PCR, 67.39 % and 17.39 % respectively (Paraoan *et al.*, 2017). The occurrence of verotoxigenic *E. coli*, *Salmonella*, and *L. monocytogenes* in surface water used for irrigation in the Lower Mainland of British Columbia was studied by Falardeau J *et al.,* (2017) through the detection of virulence genes targeting primers by multiplex PCR. Kheiri *et al.,* (2016) developed two multiplex PCR assays to simultaneously detect six water-borne bacterial pathogens such as *E. coli-uidA*, *Shigella –int*, and *P. aeruginosa- gyrB* and *Salmonella –invA, V. choleare –ompW* and coliforms-*lac Z* with a detection sensitivity of 3×10^2-3×10^3 CFU respectively.

The *lacZ* gene has been effectively employed as a target molecule to identify coliform bacteria, according to Dehghan Fatemeh *et al*., (2014). The presence of total coliform in the samples was detected after analyzing the 876 bp gene fragment. In addition, the *uidA* gene, which is found only in *E. coli,* was employed to detect

E. coli. Long incubation times, microorganism interactions, the lack of precision and sensitivity required, and poor identification of VBNC bacteria are some of the drawbacks of cultivation methods for identifying coliforms. For the identification of coliforms, molecular techniques have been proposed as an effective and quick approach. PCR will detect coliform bacteria using the *lacZ* gene (β-galactosidase gene) and *E. coli* bacteria using the *uidA* (β-glucuronidase gene) gene. The sensitivity and accuracy of new molecular approaches have been compared to traditional methods for identifying coliforms, and their practical use has been evaluated. Initial PCR screening, sample removal with negative test results, and test concentration on positive samples reduce the consumption of high media volumes and the expert's involvement in creating and removing media.

Omar and Barnard (2014) reported an mPCR assay to distinguish between pathogenic and commensal *E. coli*, from clinical and environmental water sources. To study the existence of 11 virulence genes in *E. coli*, the optimized mPCR was created, mainly *eaeA* (intimin), *bfpp* (bundle-forming pili), *stx1* (Shiga like toxin 1), *st* (heat-labile enterotoxin), *stx2* (Shiga-like toxin 2), *st* (heat-stable enterotoxin), *eagg* (enteroaggregative toxin), *ial* (invasive toxin), and *astA* (toxin EAST1). Besides, the *mdh* (malate dehydrogenase) and *gapdh* (glyceraldehyde 3-phosphate dehydrogenase) genes were used as controls to assess the false-negative results due to PCR inhibitors and the method's sensitivity (Omar & Barnard, 2014).

2.2.3. Quantitative Real-time PCR

PCR is a method that massively amplifies a single copy (or more) of a DNA sequence to produce thousands to millions of more copies of that specific DNA with the help of a DNA polymerase enzyme, which requires a free 3′-OH group provided by a primer. The primer can be either the complementary target sequence or a part of the DNA adjacent to the target sequence to which it is possible to add the first nucleotide (Mandal *et al.*, 2011).

However, PCR has some limitations such as low throughput, lack of quantification, no distinction between live and dead cells, and the reduction in sensitivity and specificity induced by post-PCR analysis. Many of these limitations are eliminated by a highly sensitive, specific real-time PCR. Quantitative real-time PCR is a robust method for determining water quality (Fumian *et al.*, 2010). PCR products are measured by detecting fluorescent signals emitted by specific dual-label probes or intercalating dyes, where the fluorescent intensity is directly proportional to the number of PCR products produced (Rompre *et al.*, 2002; Ramirez-Castillo *et al.*, 2015). SYBR Green, TaqMan probes, Molecular Beacons, Scorpion probes, and Light cycler probes are the most commonly used fluorescent systems for real-time PCR.

2.2.3.1. SYBR Green I

SYBR Green I is an asymmetrical cyanine dye that binds to double-stranded DNA by intercalating between the DNA bases,

forming a DNA-dye complex that absorbs blue light (λmax = 498 nm) and emits green (λmax = 522 nm) (Zipper *et al.*, 2004). During binding, form produces a fluorescent signal on amplification 1000 times greater than unbound form. Major advantages of SYBR Green assay include cheap ease of assay design and melt curve analysis. Furthermore, the disadvantage includes, it may generate false-positive results by binding nonspecific double-stranded DNA. SYBR Green I has been applied for the quantification of pathogens in environmental samples (McCrea *et al.*, 2007). It is used in quantitative PCR because fluorescence can be measured at the end of each amplification cycle to determine how much DNA has been amplified (Zipper *et al.*, 2004). Melting curve analysis and comparison is the method of increasing specificity of the reaction, and the melting peak will distinguish amplicons from contaminants. SYBR Green is generally used in monoplex reactions, when the connection with melting point analysis, it will help in multiplex assay also (Ririe *et al.*, 1997). The major disadvantage of SYBR Green I, it may generate false-positive results by binding nonspecific double-stranded DNA.

2.2.3.2. Hydrolytic Probes

Taqman probes are short oligonucleotides with reporter fluorophore at the 5'- end and 3'- end with a quencher molecule. Intact probes do not exhibit fluorescence because of the presence of a quencher molecule. During DNA amplification, Taqman probes hybridizes to the template strand and digested with the 5'-3' endonuclease activity of the enzyme, separating quencher from the

fluorophore, which now fluoresces and PCR products are monitored during increasing fluorescent signal (Holland *et al.*, 1991). The advantages of TaqMan probes include high specificity and the ability to perform multiplex reactions, and the disadvantage is the need for the synthesis of different probes for different sequences.

2.2.3.3. Molecular Beacons

Molecular beacons are attached with reporter fluorophore at one end and quencher at another; the amplicon sequence is complementary only to the middle part, whereas terminal nucleotides are self-complementary. In a free probe, when reporter fluorophore is attached with a quencher molecule, it forms a loop-like structure, during amplification, the stem is opened and released the quencher (Tyagi & Kramer, 1996; Kostrikis *et al.*, 1998; Tyagi *et al.*, 1998).

2.2.3.4. Lightcycler Probes

Here two hybridization probes are used, one with donor fluorophore molecule at 3' end and another with acceptor fluorophore at 5' end. During amplification, both probes hybridize with the target sequence and allow dyes near, allowing FRET. Donor dye transfers the energy, and at a different wavelength, the acceptor dye dissipates fluorescence, and the fluorescence is directly proportional to the amount of synthesized DNA (Arya *et al.*, 2005).

2.2.3.5. Scorpion Probes

These are similar to molecular beacons but serve as primer functions in PCR, having self-complementary sequences that form a 5' stem-loop structure with loop sequence complementary to amplicon sequence following the primer sequence.The stem is labeled with fluorophore and quencher molecules, respectively. During the primary step, primer extension takes place yields to a single-stranded template for the reverse primer in the second step. Then the stem opens and binds between loop and product, separating the fluorophore and quencher molecules (Whitcombe *et al.*, 1998; Whitcombe *et al.*, 1999).

2.2.3.6. Understanding CT value

A positive reaction is detected by the accumulation of the fluorescent signal in a real-time PCR assay. The Ct (cycle threshold) is defined as ‘‘the number of cycles required for the fluorescent signal to cross the threshold’’. Ct levels are inversely proportional to the sample’s amount of target nucleic acid.

Strong positive reactions indicative of the abundant target nucleic acid in the sample are Ct below or equal to 29. Positive reactions indicating moderate amounts of target nucleic acid Cts of 30-37 and Cts of 38-40 are weak reactions indicating minimal amounts of target nucleic acid that could represent an infection state or environmental contamination (Schefe *et al.*, 2006).

Baseline- Defined as ‘‘the reporter fluorescent signal is accumulating but is beneath the limit of detection of the instrument.

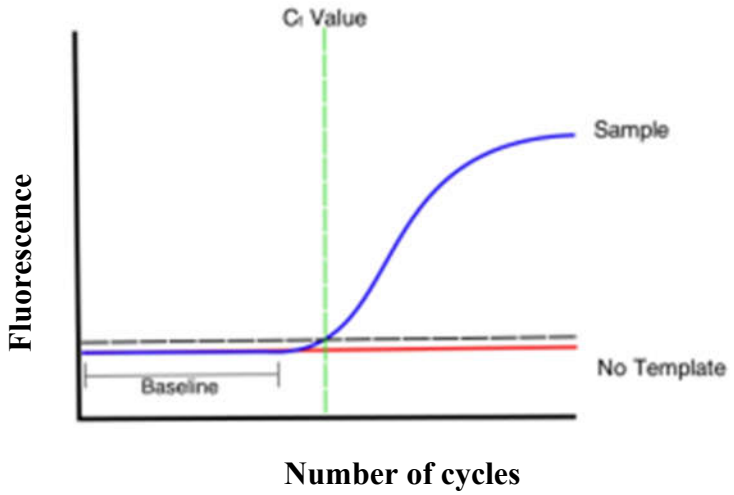


Figure 2.3. A model of a single amplification plot commonly used in real-time quantitative PCR

2.2.3.7. Quantitative analysis

The number of templates can be quantified either absolutely or relatively.

In relative quantification, changes in the number of target genes are compared with endogenous or reference control DNA. It usually uses housekeeping genes (beta actin, GAPDH, etc); it does not require standards of known concentrations. Reference genes include mRNA molecules that are endogenous or exogenous (Chelly *et al.*, 1990; Botes *et al.*, 2013), can be co-amplified with unknown targets or separately by measuring their final ratio. The relative gene expression of the target is directly proportional to the difference between threshold

cycles of housekeeping genes and the target gene (Klein, 2002). So this is not a reliable method for quantification (Orlando *et al.*, 1998).

In absolute quantification, quantification of DNA by comparing the reference material with known copy numbers requires generating the standard curve. Serial dilution of the standard is prepared, and the Ct values from each dilution are then plotted against the number of standards. Comparing the experimental Ct values with a standard curve will give the number of targets in the sample. This method is more accurate but also more laborious (Bustin, 2004)

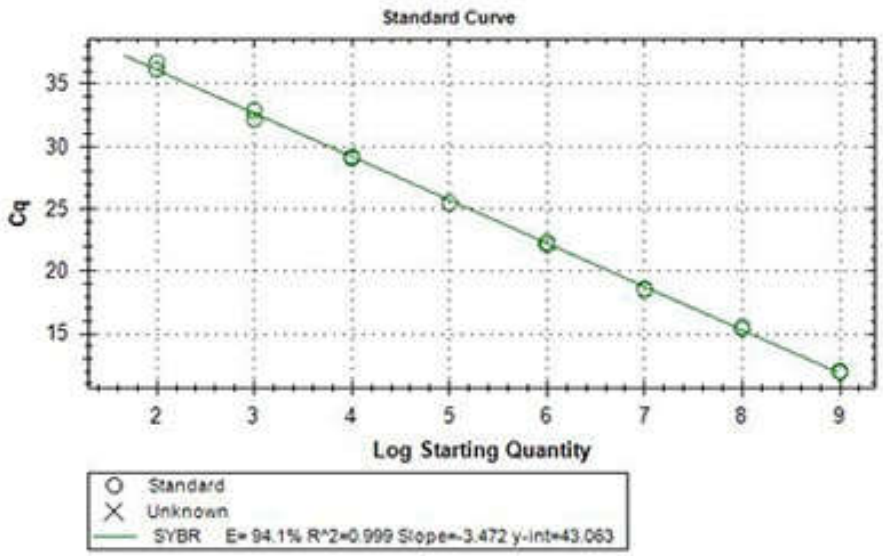


Figure 2.4. A model of a standard curve commonly used in quantification in real-time PCR

2.2.3.8. Application of real-time PCR in water-borne bacterial pathogen detection

Quantitative real-time PCR (qPCR) helps in the quantification of DNA targets by monitoring the fluorescence (Valasek & Repa, 2005), by monitoring the fluorescence with the help of dual-labeled fluorescent probes such as the TaqMan probe and the fluorescent dye SYBR green, quantitative real-time PCR (qPCR) helps quantify DNA targets. This method is highly specific and sensitive, has a high detection rate, does not require post-PCR analysis, and minimizes the risk of cross-contamination (Omiccioli *et al.*, 2009)

El –Sayed *et al.,* (2019) studied the applicability of Taqman qPCR for non-virulent *Vibrio cholerae, E. coli,* and *Salmonella enterica* in drinking water from treatment plants and tested both inlet and outlet samples respectively in Egypt by comparing TaqMan qPCR and membrane filtration methods. From the results, it was clear that in qPCR 97.96 % - 99.14 % specificity and MF it was in the range of 50 % - 91.67 %, 80 %, for *E. coli, S. enterica,* and non-virulent *V. cholerae*, respectively, and outlet samples were found to be potable and free of microbes (El-Sayed *et al.*, 2019). Many studies proved qPCR assay was a powerful tool for the detection of pathogens from culture negative environmental samples; that is why it becomes an emerging diagnostic tool. Lam *et al.,* (2014) evaluated real-time PCR to detect *E. coli* in beach water by comparing it with traditional culture methods, and it showed a significant positive linear relationship with a 0.64 correlation coefficient.

Apart from the commonly used dye for qRTPCR, a novel dye was applied by Cao *et al.,* (2019) to monitor the disinfection efficacy of *E. coli* O157: H7 in bottled water by excluding the dead cells. Depending upon the organisms and samples to be tested, pretreatment should be required in case of some samples. In water, due to the low detection limit, the membrane filtration method is used for concentration (Gibson & Schwab, 2011). Quantitative real-time PCR (qPCR) systems have proven to be a powerful tool in the field of water quality determination (Girones *et al.*, 2010). The intensity of fluorescence emitted by specific dual-labeled probes or intercalating dyes is directly proportional to the number of PCR products produced and there is no need for agarose gel electrophoresis as in traditional PCR. (Rompre *et al.*, 2002; Ramirez-Castillo *et al.*, 2015). The most commonly used fluorescent systems are SYBR Green, TaqMan Probes, and molecular beacons. There are two types of quantification, mainly absolute quantification and relative quantification.

Absolute quantification is used to quantify and express genes in an absolute manner. This implies that samples of unknown concentrations are amplified against the known standard concentrations. The absolute value is determined by the unknown sample quantification cycle (cycle of quantification, Cq) values for standards with known quantities (Leong *et al.*, 2007). The comparison of two gene levels, target gene and the reference gene, in a single sample involves relative quantification. The results are expressed as a ratio of these genes (Pfaffl, 2001). qPCR is more sensitive than

conventional methods and becoming established in the environmental sector.

In a study, Maheux and his colleagues (2013) compared mCP agar and a CRENAME (concentration and recovery of microbial particles, extraction of nucleic acids, and molecular enrichment) and *cpa* rtPCR (CRENAME + *cpa* rtPCR) for sensitive detection of *C. perfringens* spores in drinking water and results showed that by using this method, the detection rate is as few as one *C. perfringens* CFU per 100 ml and it required less than 5 h, whereas mCP agar required more than 24h. It also helps in the simultaneous detection of *E. coli* and *C. perfringens* from the same sample (Maheux *et al.*, 2013).

According to Wang Z *et al.,* (2016) a novel bacteriophage-mediated *E. coli* O157: H7 detection was reported. Conjugated with bacteriophage, carboxylic acid-functionalized magnetic beads were used to separate and concentrate *E. coli* O157:H7. The resulting complexes of bead phage-bacteria were detected quantitatively by real-time PCR.

Maheux *et al.,* (2014) targeted *LacZ, WecG*, and *16S rRNA* to detect total coliforms and *E. coli* in the water sample. Results showed *that LacZ, WecG,* and *16S rRNA* qPCR assays were detected with a sensitivity of 90.5 %, 75.5 %, and 99.3 %, respectively. Real-time mPCR has been demonstrated by Maheux *et al.,* (2014). Many studies suggest that real-time PCR is an excellent practice tool for quantitative analysis of environmental samples, and its rapidity and eases compared with traditional methods prefer real-time PCR for environmental sample analysis.

Recently, David I Walker *et al.,* (2017) designed highly specific *E. coli* qPCR using the *ybbW* gene and compared existing methods such as conventional culture methods and qNASBA for an environmental water sample. They claimed that this method was the first qPCR assay with 100 % target exclusivity.

Different single qPCR can simultaneously be run in nanolitre volume chambers on a chip in high densities in microfluidic qPCR. So it can reduce workload and time compared to the conventional one, so it can be applied in water and food samples by using the TaqMan probe labeled with various fluorophores that can specifically detect *L. monocytogenes, V. cholerae, S. typhimurium, S. flexneri, C. perfringens, V. parahaemolyticus, Pseudogulbenkiana spp*., and pathogenic *E. Coli.* Ishii and Co-workers (2013) established a microfluidic qPCR with a limit of 100 cells/L detection. Many studies have revealed that the quantification of pathogens using real-time PCR showed higher sensitivity than pure culture (Sharma, 2003; Alhamlan *et al.*, 2015).

A microfluidic qPCR array chip was designed for the multiplex assay detecting *A. hydrophilia, K. pneumoniae, S. aureus,* and *P. aeruginosa,* respectively (Ramalingam *et al.*, 2010). A novel nanofluidic RT-qPCR method was a successive tool for quantifying enteric viruses (Coudray-Meunier *et al.*, 2016; Monteiro & Santos, 2017).

CHAPTER 3

DETECTION OF FECAL COLIFORMS AND PREVALENCE OF OTHER WATERBORNE PATHOGENS

3.1. Introduction

Many diseases are reported annually due to unsafe drinking water because of a lack of access to safe drinking water and poor sanitation (Hunter *et al.*, 2001). The United Nations identifies water quality improvement as one of the eight (MDGs) and aims to reduce by 50 % the number of people without access to safe water in 2015 (Pandey *et al.*, 2014). The global burden of waterborne diseases is Millennium Development Goals still significant, although waterborne outbreaks have decreased dramatically since the 1900s. Moreover, the number of outbreaks underestimates the actual incidence of waterborne diseases (Leclerc H *et al.*, 2002). So there is an urgent need to take any action to control the cases of waterborne diseases. In India, contaminated water consumption plays a vital role in many waterborne disease outbreaks (Joseph *et al.*, 2018). Coliforms are major contaminants in surface and groundwater in developing countries and represent an essential group of indicator bacteria as a measure of water quality (Chitanand *et al.*, 2010).

Ground water is the primary source of drinking water, and the quality of water is threatened by several parameters, including microbiological and chemical contamination (Kolbel-Boelke *et al.*, 1988). A significant source of microbial pathogens in developing regions is drinking water. Waterborne infections are transmitted through ingestion, airborne or direct contact by infectious agents such as bacteria, viruses, protozoa, and helminths. As a result of their phenotypic plasticity, heterotrophic bacteria are mainly prevalent in groundwater. Groundwater examination shows the prevalence of

Pseudomonas spp. in many samples (Leclerc, 2003). When there is fecal or other contamination, the dominance of pathogenic bacteria increases.

The introduction of coliforms in drinking water may come from sewage systems, so water analysis mainly focuses on coliforms, thermotolerant coliforms and *E. coli* (Tantawiwat *et al.*, 2005). *E. coli* is considered as the typical fecal indicator. Fecal coliforms are thermotolerant can ferment lactose at 44.5 °C (Craun, 1978; Grabow, 1996; Rompre *et al.*, 2002; Payment *et al.*, 2003). And the presence of fecal coliforms indicates recent contamination of water sources with human and animal wastes and these 'indicator organisms' indicate the possible presence of other potential pathogens (Cabral, 2010). Total coliforms are Gram-negative bacilli, oxidase-negative, non sporing bacteria and are facultative anaerobes ferment lactose with gas production at 35–37 °C, after 48 hours and it comprised of *Escherichia*, *Klebsiella*, *Enterobacter,* and *Citrobacter.* But the significance of total coliforms as sanitary significance is disparate because it also contains soil and vegetation. There is no relation between total coliform count and fecal pollution. Strict governmental regulations are applicable to the use of the coliform group as an indicator of fecal contamination. Among the intestinal flora of warm-blooded animals, *E. coli* is the major coliform. Its presence is associated with fecal contamination, so no *E. coli* is allowed in drinking water. The detection of indicator organisms is therefore considered the best way to detect the efficacy of the disinfection

process as well as recent and frequent fecal water contamination (Tharannum *et al.*, 2009; Rodríguez *et al.*, 2012).

This chapter initiated to assess the microbiological quality of drinking water by using conventional methods. Many conventional culture-based techniques have been used to cultivate and identify target pathogens, such as multiple-tube fermentation techniques and standard bacterial cell culture techniques. Following presumptive, confirmed, and completed tests, the tubes are interpreted as the most probable number (MPN) and help estimate total coliforms in the sample and viable culture count allows the growth and enumeration of viable cells bacteria.

3.2. Materials and Methods

3.2.1. Sampling

Sixty drinking water samples from different sources (well water, bore well water and public water supply) were collected aseptically for one year. Bacteriological examination of water samples was carried out immediately after collection.

Heterotrophic plate count (standard plate count) was performed to measure the overall bacteriological quality of drinking water. And for the isolation of total coliforms, a multiple tube fermentation method (MPN) was used. Enteric bacteria isolated on respective selective or differential media were identified based on their colonial,

morphological, and biochemical characteristics following Bergey's Manual of determinative Microbiology (Bergey, D.H.& Holt., 1994).

3.2.2. Quantitative analysis through total viable count

Serial dilution of the test water samples was made with sterile distilled water. 0.1 ml of the test samples from 10^{-1} to 10^{-6} dilutions were dispensed into the Petri dishes with sterile nutrient agar medium. The analysis was performed in duplicates (Cappuccino & Sherman, 1996). Plates were incubated at 37 °C for 24 hours. After incubation, CFU/ml was calculated and isolated colonies were subcultured on to nutrient agar plates. For the final identification, all the isolates were identified by using primary as well as secondary identification methods such as Gram's staining, biochemical methods according to Bergey's Manual of determinative Microbiology (Bergey, D.H.& Holt., 1994).

3.2.3. Most probable number method using 5 tube method for the detection of total coliforms (Cappuccino & Sherman, 1996).

Prepared double strength phenol red lactose broth (DSLB) and single strength phenol red lactose broth (SSLB) with Durham’s tubes. Five tubes of 10 ml DSLB were inoculated with 10ml sample and other five tubes sets of 9 ml SSLB tubes added by 1ml of sample and 9.9 ml tubes were added by 0.1 ml samples**.** Incubated the tubes at 37 °C for 24-48 hours. After incubation checked the production of acid and gas. Noted the numbers of the positive tubes and comparing the result with McCrady’s table.

In confirmed tests, the positive tubes were selected from the MPN test and streaked on to the EMB agar. Incubated the plates at 37 °C at 18-24 hours. Colony morphology with characteristic green metallic sheen in the media were inoculated into brilliant green lactose bile broth for fermentation and nutrient agar. In completed tests, they were incubated at 37 °C for overnight and tested for the biochemical properties (IMViC Tests) and Gram's staining and checked the production of acid and gas in lactose broth.

3.2.4. Selective isolation of waterborne pathogens

Protocols for isolating and identifying *E. coli, S. enterica, Shigella spp., V. cholerae, P. aeruginosa*, *Y. enterocolitica,* and *A. hydrophila* are available in Bergey's Manual of determinative Microbiology (Bergey, D.H.& Holt., 1994).

3.2.4.1. Pre enrichment

In pre-enrichment media, the pathogenic organisms were cultured. The step will increase the sensitivity and reliability of the isolation just before the pathogens are selectively isolated and improve the detection of the selected pathogens. Such media can provide the best growth, and appropriate conditions for injured/metabolically harmed organisms to grow. 50 ml of water was filtered through 0.45µm nitrocellulose filter paper from the collected samples and transferred to the corresponding enrichment broth cultures. Then the cultures were inoculated on selective agar plates after incubation. Table 3.1 shows the details of the pre-enrichment media.

Table 3.1. Pre-enrichment conditions of selected pathogens

Organisms	Pre-enrichment Media	Growth conditions
E. coli	Nutrient broth (NB)	37°C /18-24 hours.
Pseudomonas spp	Nutrient broth (NB)	37°C / 18-24 hours.
Salmonella spp	Buffered peptone water	37°C / 18-24 hours.
Shigella spp	Nutrient broth (NB)	37°C / 18-24 hours.
V. cholerae	Alkaline peptone water	37°C / 18-24 hours.
A. hydrophila	Alkaline peptone water	37°C / 18-24 hours.
Y. enterocolitica	Yersinia enrichment broth	10°C / 10 days

3.2.4.2. Selective media

The tubes were taken from pre-enriched cultures and the inoculum was streaked into the respective selective media to isolate the specific pathogens (as given in Table 3.2) and incubated under appropriate conditions.

Table 3.2. Selected pathogens and their growth conditions

Organism	Selective media	Growth conditions
E. coli	Eosin-Methylene-Blue agar (EMB)	37°C / 24-48 hours.
Pseudomonas spp	King's B agar	37°C / 24-48 hours.
Salmonella spp	Deoxy Cholate Agar (DCA)	37°C / 24-48 hours.
Shigella spp	Deoxy Cholate Agar (DCA)	37°C / 24-48 hours.
Vibrio spp	Thiosulfate Citrate Bile Salt Sucrose Agar (TCBSA)	37°C / 24-48 hours.
Aeromonas spp	Starch Ampicillin Agar (SAA)	30°C / 24-48 hours.
Yersinia spp	Yersinia selective agar (YSA)	30°C / 24-48 hours.

3.2.5. Identification of isolates using conventional methods (Cappuccino & Sherman, 1996)

3.2.5.1. Gram staining

Prepared the bacterial smear and heat fixed it. Then floated smear with crystal violet stands for 1 minute. Washed with running tap water. After that, the smear was flooded with gram's iodine as a mordant for 1 minute, and then washed with running tap water and decolorizer (95 % alcohol). Then the smear was counterstained by saffranine and waited for 45 seconds. Finally, washed the slide with water and blot dried with bibulous paper and observed under oil immersion.

3.2.5.2. Biochemical identification

a). Catalase test

Took a clean glass slide and placed a drop of 3 % of hydrogen peroxide (H_2O_2). A small portion of the bacterial colony was taken and emulsified in the dropped by used nonmetallic stuck. Then observed the effervescence of gas bubbles.

b). Oxidase test

Readymade oxidase disc was placed on a clean glass slide and bacterial culture was placed on the top of the disc. The disc was observed for immediate color changed.

c). Indole production test

Used sterile technique, the bacterial culture was inoculated into about 5 ml of tryptone broth at 37 °C for 24 hours. Then added 0. 5 ml

of kovac's reagent into the culture and observed for the cherry red layer formation.

d). Methyl red (MR) test

The test organisms were inoculated on to MR-VP medium and incubated at 37 °C for 24 hours. The result was observed by adding a 2-3 drops of methyl red solution.

e). Voges- Proskauer (VP) test

Test organism was inoculated into the sterile tubes of the MR-VP medium. Then incubated the culture at 37 °C for 24 hours. After incubation, 3 ml of barritt's reagent 'a' and barritt's reagent 'b' was added. Mixed well and aerated at intervals and results was observed up to 30 minutes for color formation.

f). Citrate utilization test

Prepared simmon's citrate slants, inoculated the test organisms by used sterile techniques and incubated the tubes at 37 °C for 24 hours. The results were observed as a color changed in the inoculating medium.

g). Triple sugar iron agar (TSI) test

Used aseptic techniques, test organisms were inoculated onto triple sugar iron agar through stabbed and streak inoculation. Then the tubes was incubated at 37 °C for 18-24 hours and observed for the changes in the medium.

h). Urease test

Christensen's agar slants were prepared and inoculated the bacterial culture by streaking and incubated at 37 °C for 18-24 hours. After incubation, slants were observed for the typical color changed and growth of the organisms.

i). Nitrate reduction test

The test organisms were inoculated onto nitrate broth and incubated at 37 °C for 24 hours. After incubation, added nitrate reagent 'a' and 'b' and then observe the red color as a positive test. If the test doesn't produce a red color, then the test could been continued by adding zinc powder.

j). Carbohydrate fermentation test

Prepared the media with suitable carbohydrates such as glucose, lactose, sucrose, and mannitol. Added phenol red as a pH indicator for the fermentation. The test tubes with appropriate media were inoculated with samples and incubated at 37 °C for 18-24 hours with Durham’s tubes. The results were noted by the changed of color of the medium and gas bubble formation.

k). MacConkey agar

The major constituent of the medium was lactose. Many bacteria could ferment lactose they produced pink colonies in MacConkey media. MacConkey agar plates were prepared and streaked by the samples. Characteristic changes were observed.

l). Bile -Esculin agar test

This test was used to identify group D streptococcus (*Enterococcus spp*). The bile esculin tubes/plates was inoculated with samples and incubated at 35-37 °C for 24 hours. A black color noted the result in the medim, which was produced by the hydrolysis of the esculin.

m). Mannitol salt agar test

Mannitol salt agar was a well-known selective media of *Staphylococcus aureus* and also *S. epidermidis* and micrococci could grow on it. Prepared the MSA medium and poured it into petri dishes. The samples were streaked on the agar plates and incubated overnight at 37 °C. The positive result would alter the pHof the medium and give a yellow color colony.

n). Coagulase test

Distinguish coagulase positive *Staphylococcus aureus* from Coagulase Negative *Staphylococcus* (CONS) coagulase test is used. Coagulase is the enzyme produced by *S. aureus*, which is mainly 2 types, bound and free, transforming soluble plasma fibrinogen to insoluble fibrin. Bound coagulase can be detected with the help of slide coagulase test and free coagulase by tube test.

- ***Slide Coagulase Test***

Smooth milky white suspensions of bacteria were prepared in the physiological saline; citrated plasma was added and immediately observed for clumping within 10 seconds.

- ***Tube coagulase Test***

To detect the free coagulase, which reacts with the coagulase reacting factor, convert fibrinogen to fibrin. To the 1:10 diluted plasma, organism suspensions were inoculated and kept at 37 °C for 1 hour, and results were observed for up to 4 hours.

3.3. Results

3.3.1. Most probable number method for the detection of fecal coliforms

Water quality analysis was carried out using the most probable number method to detect total coliforms, including presumptive, confirmed, and completed tests. The presumptive coliform counts of the test samples were in the range of 0- >1600/100 ml and many of the samples exceeded the WHO standard limit (0 coliform/100 ml) (WHO, 1993) and data presented in Table 3.1. *E. coli* was further confirmed using confirmed and completed tests. In our study, about 46.7 % of the samples were in the category of excellent, followed by 1.6 % in satisfactory, 6.7 % in suspicious and 45 % of the samples were in nonpotable unsatisfactory results.

3.3.2. Quantitative analysis through total Viable Count

The standard plate count which indicates total microbial count in drinking water helps in the quantitative analysis using the determination of CFU (Colony-forming unit), using this equation,

$$N = \frac{C}{V\left(n_1 + 0.1n_2\right) d}$$

*Experiment done in duplicates.

C – Sum of colonies on all plates

n_1 – Number of plates in the first dilution

V – Volume of sample plated

n_2 – Number of plates in the second dilution

d – Dilution factor

The total microbial count in drinking water samples was in the range of 90 to $8x10^6$ CFU/ml.The results are in Table 3.3.

Table 3.3. Quantitative determination of waterborne pathogens

Sl. No	Positive Tubes			MPN index	95 % confidence limits		CFU/ml
	10ml	1ml	0.1ml	/100ml	Lower	Upper	
1	0	0	0	0	-	5.4	18×10^3
2	5	5	5	>1600	800	-	76×10^5
3	3	2	0	14	4.8	35	86×10^3
4	5	3	1	110	30	280	28.1×10^4
5	0	0	0	0	-	5.4	1.9×10^3
6	5	1	0	33	8.9	120	11.8×10^4
7	0	0	0	0	-	5.4	90
8	0	0	0	0	-	5.4	13.6×10^3
9	5	5	5	>1600	800	-	3.5×10^6
10	0	0	0	0	-	5.4	2.13×10^3
11	3	2	0	14	4.8	35	36×10^3
12	0	0	0	0	-	5.4	2×10^3
13	4	1	1	21	6.2	44	84×10^3
14	5	4	2	220	62	490	56×10^4

15	0	0	0	0	-	5.4	5×10^2
16	2	1	0	6.8	0.55	17	1.3×10^3
17	4	1	1	21	6.2	44	4.7×10^3
18	0	0	0	0	-	5.4	9.7×10^2
19	5	4	3	280	88	750	83×10^4
20	0	0	0	0	-	1.3	7×10^2
21	5	5	3	920	210	3000	6.3×10^6
22	0	0	0	0	-	5.4	5×10^2
23	5	0	2	43	12	120	76×10^3
24	1	0	1	4	0.49	12	2.1×10^3
25	4	0	1	17	5	36	4.9×10^3
26	4	0	0	13	3.3	35	6.3×10^3
27	0	0	0	0	-	1.3	190
28	5	3	1	110	30	280	88×10^4
29	5	2	2	95	29	240	15.1×10^4
30	0	0	0	0	-	5.4	96
31	0	0	0	0	-	5.4	810
32	5	4	3	280	88	750	8.4×10^5
33	0	0	0	0	-	5.4	450
34	5	5	3	920	210	3000	2.7×10^6
35	4	3	0	27	8.6	69	46×10^3
36	0	0	0	0	-	5.4	3×10^3
37	5	5	5	>1600	800	-	8×10^6
38	0	0	0	0	-	5.4	1.3×10^3
39	0	0	0	0	-	5.4	9.7×10^2
40	5	5	2	540	130	2000	5.1×10^6
41	0	0	0	0	-	5.4	1×10^3
42	4	0	0	13	3.3	35	45×10^3
43	0	0	0	0	-	5.4	7×10^3
44	1	0	0	2	0.052	12	98×10^2
45	4	3	0	27	8.6	69	28.4×10^4
46	0	0	0	0	-	5.4	3×10^3
47	0	0	0	0	-	5.4	14×10^2
48	0	0	0	0	-	5.4	12×10^3
49	1	1	0	4	0.49	13	7.5×10^3
50	0	0	0	0	-	5.4	5×10^3
51	5	5	0	240	65	740	74.5×10^4
52	0	0	0	0	-	5.4	6.1×10^2
53	0	0	0	0	-	5.4	130

54	4	3	0	27	8.6	69	21×10^3
56	0	0	0	0	-	5.4	365
57	0	0	0	0	-	5.4	111
58	0	0	0	0	-	5.4	4×10^2
59	5	1	0	33	8.9	120	8.9×10^3
60	3	1	1	14	4.7	35	11×10^3

Based on the presumptive coliform count, the quality can be measured, and the grading of the samples shown in Table 3.4.

Table 3.4. Presumptive coliform count vs Grade of water

Grade of water sample	Presumptive coliform count/100ml	Number (%) of water samples (n=60)
Excellent	0	46.7
Satisfactory	01-03	1.6
Suspicious	04-10	6.7
Unsatisfactory	>10	45

3.3.3. Selective isolation of target pathogens

After membrane filtration of the samples, it was pre enriched in the corresponding broth medium and, after overnight incubation, streaked into selective media and incubated. The overall picture of the results is as follows.

Table 3.5. Selective isolation of target pathogens

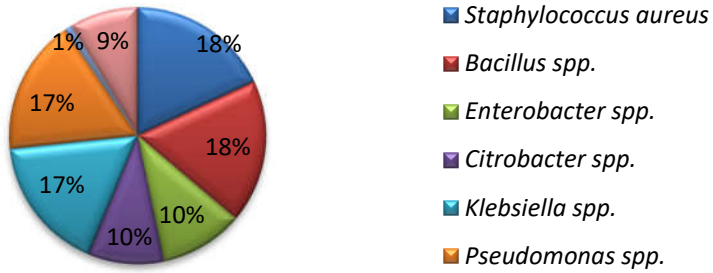
Selective media	Pathogens	Interference	Positive samples
Eosin methylene blue (EMB)agar	*E .coli*	Large, blue-black colonies with a green metallic sheen	Positive
King's B agar	*Pseudomonas spp*	Greenish-yellow colonies	Positive
Deoxy Cholate agar (DCA)	*Salmonella spp* *Shigella spp*	Colorless colonies with or without black color	Nil Positive
Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar	*V. cholerae*	Yellow colonies	Nil
Starch ampicillin agar (SAA)	*Aeromonas spp*	Produced honey color colonies surrounded by clear zonewithLugol's iodine	Nil
Yersinia selective agar (YSA)	*Yersinia spp*	Dark pink centered colonies	Nil

E. coli, Pseudomonas spp, and *Shigella spp.* were found to be present in culture based methods, however *Salmonella spp, V. cholerae, Aeromonas spp,* and *Yersinia spp.* were not detected in any of the samples by culture based methods (Table 3.5).

3.3.4. Prevalence of waterborne pathogens in the study area

Based on Bergey's Manual of determinative Microbiology (Bergey, D.H.& Holt., 1994), the isolates obtained from these samples were categorized. And a total of 105 bacterial isolates comprised of eight bacterial species were identified. The organism isolated were found to be *Staphylococcus aureus* (18.1 %) *Bacillus spp* (18.1 %), *Pseudomonas spp* (17.14 %), *Klebsiella spp* (17.14 %), *Enterobacter spp* (10.48 %), *Citrobacter spp* (9.52 %), *E. coli* (8.57 %), and *Shigella spp* (0.95 %) (Figure 3.1).

Figure 3.1 Prevalence of isolated organisms



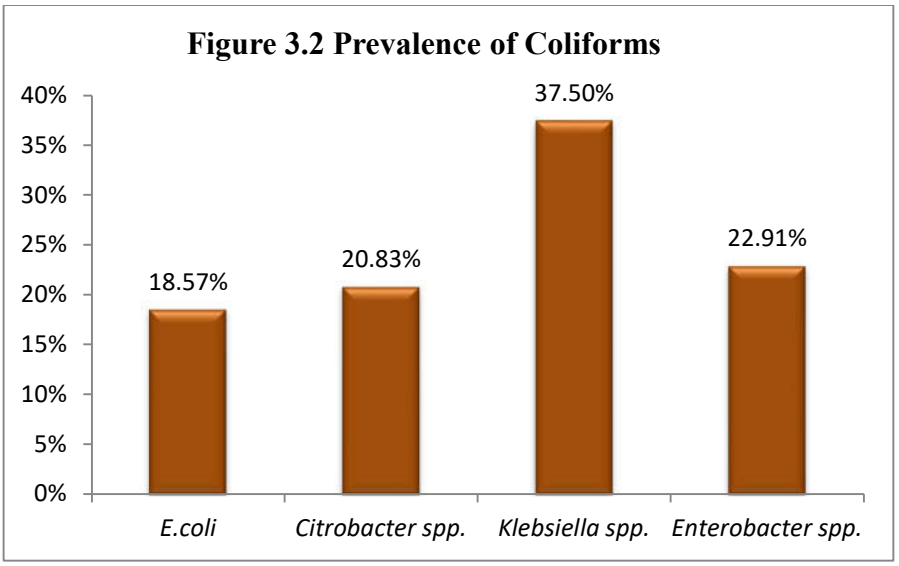
Isolates were identified according to Bergey's manual, the biochemical characteristics of the isolates were in Table 3.6.

Table 3.6. Biochemical characteristics of isolated organisms

Sl.No	GS	Cat	OX	MT	IMViC	CF				TSI	U	Nt	SPECIES
						G	L	S	M				
1	GNB	+	-	M	- -++	+	+	+	+	K/A Gas	-	+	*Enterobacter spp*
2	GPC	+	-	-	-+++	+	+	+	+	A/A	-	+	*Staphylococcus spp*
3	GNB	-	-	M	++--	+	+	+	+	A/A	-	+	*E.coli*
4	GNB	+	-	M	-+-+	+	+	+	+	A/AH_2S	+	+	*Citrobacter spp*
5	GNB	+	+	M	---+	-	-	-	-	K/NC	-	+	*Pseudomonas spp*
6	GPB	+	-	-	-+++	+	+	+	+	A/A	-	-	*Bacillus spp.*
7	GNB	+	-	NM	-+--	+	-	-	+	K/A	-	+	*Shigella spp*
8	GNB	+	-	NM	- -++	+	+	+	+	A/A	+	+	*Klebsiella spp*

Out of the 60 samples screened, 36 (60 %) were positive for the presence of coliforms (number of coliforms=48). Among which *E. coli* accounts to be 18.75 %, *Citrobacter spp* (20.83 %), *Enterobacter spp*

(22.91 %), and *Klebsiella spp.* (37.5 %) respectively (Figure 3.2). The indicator organism *E. coli* was present in a total of 9 samples among which 5 undergoes well water samples and 4 tapwater, and in borewell water no *E. coli* was found.



3.4. Discussion

Water that is used for human consumption should be free from microbial as well as chemical pollutants. It is challenging to detect all possible bacteria that might be present. The presence of indicator organisms indicates fecal contamination and the possible presence of other pathogens (WHO, 1996). Microbial contamination in water is either due to failure in disinfection processes or contamination with sewages etc. So every water supply should be regularly monitored, which helps prevent infections and protects the source from further pollution (Awoyemi *et al.*, 2014). No single approach for drinking

water standards is globally accepted, which means they have their own standards for every region. Microbial water quality may vary rapidly from time to time.

Total viable count in water analysis is a technique that helps quantitatively estimate microbial concentration in a sample. The count represents as Colony-forming Units (CFU) per ml. There are no universally acceptable concentrations of organisms, and the most allowable concentrations used by water supply agencies, health departments, and local judiciaries may vary from 100 CFU to 500 CFU/ml. The overall picture revealed that the total microbial count in drinking water samples was in the range of 90 to $8x10^6$ CFU/ml. The data shows a high fluctuation in the distribution of heterotrophic populations in the study period (Table 3.3). The high count may be associated with increased nutrient availability. Using heterotropic bacteria, drinking water quality is monitored (Grabow, 1996). These organisms are under the natural microbiota of water and usually are not pathogenic (Leclerc, 2003). But in the case of immune-suppressive patients, it may cause severe life-threatening diseases. The main participants in standard plate count include *Pseudomonas, Bacillus, Streptomyces, Proteus, Micrococci, Flavobacterium,* and various yeasts (Geldreich *et al.*, 1972; Geldreich, 1973). Fecal coliform suppression by large populations of organisms is usually seen in standards plate counts. That means 1000 non-coliforms/ml could suppress the growth of coliforms.

Most Probable Number /100 ml estimate the number of total coliforms. According to the presumptive test, water samples were categorized as excellent, unsatisfactory, etc. Central Pollution Control Board gives a standard of MPN/100ml, 50 or less in drinking water. In the case of treated water, positive MPN indicates the failure of treatment, breakthrough or contamination with sewages, etc. (Geldreich *et al.*, 1972; Clark *et al.*, 2011). The presumptive coliform count of the test samples was in the range of 0->1600/100ml, and many of the samples exceeded the WHO standard limit (0 coliform/100 ml) and CPCB standards. Jyothilekshmi *et al.*, (2019) evaluated well water samples from Alappuzha, found that MPN/100 ml was in the range of 23-2400.

The prevalence of various bacterial isolates in drinking water samples is shown in figure 3.1. *E. coli* is the crucial indicator organism associated with the sanitary quality of drinking water (Levy *et al.*, 2012). The presence of *E. coli* indicates the possible presence of other pathogens, which are the fecal origin; that is why *E. coli* is considered the indicator for detecting fecal contamination (Edberg *et al.*, 2000; Odonkor & Ampofo, 2013). In our study, the presence of *E. coli* was reported (9 %) indicates recent fecal contamination.

The highest population was *Bacillus spp*. and *Staphylococcus aureus* with 18 %. *Bacillus spp*. is considered a natural inhabitant of soil and water and readily detected in most drinking water samples as the central part of SPC bacteria due to resistance of spores to the disinfection, and they have no clinical significance. Then it was

followed by *P. aeruginosa* and *Klebsiella* spp (17 %), *Enterobacter,* and *Citrobacter* (10 %). Due to the ease of colonization and biofilm production, *Pseudomonas* is ubiquitous in water systems. Usually, these isolated organisms from these samples tested are considered nonpathogenic (WHO, 2006; Aquachem., 2009). Except for *Shigella spp* (1%), but in the case of immune compromised patients, it may result in severe diseases.

Agwaranze *et al.,* (2017) evaluated the prevalence of bacterial species in well water, where *Staphylococcus aureus* was the highest (53.33 %) and followed by *E. coli*, *Pseudomonas* species, *Proteus* species, *Salmonella* species, *Enterobacter* species, *Klebsiella* species, and *Enterococcus* species. Almost similar results were observed in our investigation.

According to figure 3.1, *Staphylococcus aureus*, *Bacillus spp*, *Pseudomonas spp*, *Klebsiella spp*, *Enterobacter spp*, *Citrobacter spp*, *E. coli*, and *Shigella spp* were the possible pathogens which is prevalent in the study area, and chapter 4 introduces development of rapid detection methods for the co- detection and enumeration of several waterborne pathogens using multiplex PCR and qPCR.

3.5 Conclusion

Drinking water should be protected from unwanted human influences. This study concluded that the standard plate count was in the 90 to $8x10^6$ CFU/ ml range, and 60 % of the samples were positive for coliforms. The prevalence of *E. coli* in these tested samples showed

9 %; this shows recent fecal contamination and may imply the potential presence of other pathogens. The results showed that some sources were faecally polluted, with the potential risk of waterborne diseases. So to prevent waterborne diseases and outbreaks, it is recommended that these water sources be routinely monitored and take necessary action for proper treatment. Further development of multiplex PCR and qPCR based rapid detection methods can help the proper monitoring of drinking water samples.

CHAPTER 4

DEVELOPMENT OF MULTIPLEX AND qPCR ASSAYS FOR THE SIMULTANEOUS DETECTION OF SELECTED WATERBORNE PATHOGENS

4.1 Introduction

Microbial water contamination is a significant public health problem leading to large-scale water-borne diseases and outbreaks (Szewzyk *et al.*, 2000). Water-borne illnesses are frequently reported even in developed countries that maintain strict water quality standards. US Centre for Disease Control, confirmed about 33 and 81 outbreaks among 1040 drinking water-related and 1326 recreational water-related diseases, respectively (CDC, 2013; Hlavsa *et al.*, 2014). In 2017, 71 % of the global population used an adequately managed drinking-water service and was free from contamination. There is even a lack of basic drinking water services for 785 million people, including one hundred and forty-four million people dependent on surface water. At least 2 billion people worldwide use a drinking water source contaminated with feces (WHO, 2017). Water-borne diseases are more prevalent in low and middle-income countries such as Africa and Southeast Asia (Johansson *et al.*, 2012).

In India, annual cases of 37.7 million people affected with water-borne diseases and 1.5 million children die of diarrhea, which leads to an annual economic burden of $600 million. About 10,738 deaths have occurred due to water-borne diseases over the last five years since 2017. Acute bacterial diarrheal infections become the most critical illness followed by viral hepatitis and cholera. The state-wise picture shows Uttar Pradesh recorded with the highest death followed by West Bengal, Assam, Odisha, and Madhya Pradesh. Open

defecation and lack of proper sewage disposal mechanisms increase the chances of infection (Central Bureau of Health Intelligence, 2018).

Direct PCR analysis of some bacteria from water is troublesome because of the sample's low number of these organisms. Hence, a pre-enrichment step is required before performing PCR from environmental samples. Molecular approaches, including quantitative PCR, offer many advantages over conventional culture-based methods for identifying and enumerating bacterial pathogens. It is also tricky, time-consuming, and costly in cases where screening for multiple targets. The use of multiplex PCR enables the rapid simultaneous detection of several water-borne pathogens (Kheiri *et al.*, 2016; Halder *et al.*, 2018; Li *et al.*, 2019). In quantitative real time PCR, the PCR products are measured by detecting fluorescent signals emitted by specific probes or intercalating dyes, where the rate of fluorescence is proportional to the number of PCR products formed (Rompre *et al.*, 2002; Ramirez-Castillo *et al.*, 2015). Quantitative PCR offers many advantages over conventional culture-based methods for the specific identification and enumeration of bacterial pathogens. Health authorities can adopt these techniques to improve water quality monitoring (Lam *et al.*, 2014; Ahmed *et al.*, 2018; El-Sayed *et al.*, 2019). Even though various methods for detecting water-borne pathogens have been developed in recent years, limited attention has been paid to developing a PCR-based rapid method for the co-detection of the seven pathogens included in this study.

This chapter presents two new approaches for the co-detection and enumeration of water-borne pathogens such as *Aeromonas hydrophila, Pseudomonas aeruginosa, Salmonella enterica, Yersinia enterocolitica, E. coli, Vibrio cholerae* and *Shigella spp* using multiplex PCR and quantitative real time PCR.

4.2. Materials and Methods

4.2.1. Culture conditions and DNA Extraction

The bacterial isolates analyzed in this study are shown in Table 4.1. Sources included clinical and environmental isolates obtained from Microbial type culture collection (MTCC), American Type Culture Collection (ATCC), and isolates from drinking water samples. The bacterial strains were grown in Luria – Bertani broth (LB broth) (Hi-Media, Mumbai, India) at 37 °C with shaking. The exception was *Y. enterocolitica*, grown in tryptic soy broth yeast extract medium at 28 °C for 48 hours. DNA extraction from standard strains was carried out by using the modified Chen *et al.,* (2012) method. For this, 1 ml of bacterial cultures were centrifuged at 5, 000 g for 5 minutes, the supernatant was discarded, and the pellet was resuspended in 180 µl of lysis buffer (0.1M Tris HCl pH (8.5)-10ml, 0.5M EDTA-1ml, 5 % lysozyme-0.5ml, 10 % SDS-2ml, 5M NaCl-4ml, 2 % proteinase K-0.5ml /100ml) and incubated at 37 °C for 1hour. Boiled for 20 minutes after incubation and centrifuged at 5,000 g for 5 minutes. An equal amount of isopropanol was added to the supernatant and centrifuged for 10 minutes at 12,000 g. Pellet was desalted with 200 µl of ice-cold

ethanol (70 %), centrifuged at 3,000 g for 5 minutes, and dry at the incubator. About 100 µl of Tris –EDTA (TE) buffer was added and stored at -20 °C until further analysis.

4.2.2. Target sequences and oligonucleotide primers

The sequences encoding virulent and species-specific genes like invasion plasmid antigen H (*IpaH*), β-glucuronidase (*uidA*), outer membrane protein (*ompW*), attachment invasion locus (*ail*), gyrase B (*gyrB*), invasion plasmid antigen B (*ipaB*) and haemolysin gene (*ahh1*) were used to amplify *Shigella spp., E. coli, V. cholerae, Y. enterocolitica, P. aeruginosa, S. enterica* and *A. hydrophila* respectively. The oligonucleotide primers (Table 4.2) were selected based on the previous literature, were synthesized by Indus Biosolutions, Bangalore, India.

4.2.3. Monoplex and multiplex PCR optimization

The primers selected from previous studies (Wang *et al.*, 2003; Fan *et al.*, 2008; Balakrishna *et al.*, 2010; Lee *et al.*, 2011; Mehrabadi *et al.*, 2012; Babu *et al.*, 2013) based on their specificity and sensitivity. The composition of the PCR reaction mixture is given in Table 4.3. The PCR was performed under the following conditions by the Bio-Rad, T^{100} thermal cycler: 94 °C for 5 minutes, 40 cycles of 94 °C for 30 seconds, 60 °C for 1 minute for all targets except *Yersinia spp* 52 °C for 1 minute, 72 °C for 1 minute, followed by final extension at 72 °C for 10 minutes (Table 4.4). The PCR amplicons were analyzed on 2 % agarose gel with 0.5 µg/ml ethidium bromide

and further visualized using the Bio-Rad Gel Doc 2000 documentation system.

Table 4.1. The bacterial strains used for the specificity assessment of the primers

Strain	Source	*Ahh1*	*GyrB*	*IpaB*	*Ail*	*UidA*	*OmpW*	*IpaH*
Aeromonas hydrophila	MTCC 1739	+	-	-	-	-	-	-
E. coli	MTCC1687	-	-	-	-	+	-	-
E. coli	Clinical strain	-	-	-	-	+	-	-
E. coli	ATCC 25922	-	-	-	-	+	-	-
E. coli	Environmental strain	-	-	-	-	+	-	-
Pseudomonas aeruginosa	MTCC 2453	-	+	-	-	-	-	-
Pseudomonas aeruginosa	Clinical strain	-	+	-	-	-	-	-
Pseudomonas aeruginosa	ATCC 27853	-	+	-	-	-	-	-
Pseudomonas aeruginosa	Environmental strain	-	+	-	-	-	-	-
Pseudomonas aeruginosa	Lab strain	-	+	-	-	-	-	-
Pseudomonas putida	Clinical strain	-	-	-	-	-	-	-
Klebsiella spp.	MTCC 109	-	-	-	-	-	-	-
Klebsiella spp.	Clinical strain	-	-	-	-	-	-	-
Klebsiella spp.	Environmental strain	-	-	-	-	-	-	-
Klebsiella spp.	Lab strain,	-	-	-	-	-	-	-
Enterobacter spp.	Environmental strain	-	-	-	-	-	-	-

Enterobacter spp.	Clinical strain	-	-	-	-	-	-	-
Staphylococcus aureus	MTCC 737	-	-	-	-	-	-	-
Staphylococcus aureus	ATCC 29213	-	-	-	-	-	-	-
Staphylococcus aureus	Environmental strain	-	-	-	-	-	-	-
Staphylococcus aureus	Clinical strain	-	-	-	-	-	-	-
Staphylococcus aureus	Lab strain	-	-	-	-	-	-	-
Bacillus subtilis	Environmental strain	-	-	-	-	-	-	-
Bacillus subtilis	Lab strain	-	-	-	-	-	-	-
Bacillus thuringenesis	Standard strain	-	-	-	-	-	-	-
Citrobacter spp.	Environmental strain	-	-	-	-	-	-	-
Bacillus subtilis	Clinical strain	-	-	-	-	-	-	-
Shigella spp.	Clinical strain	-	-	-	-	-	-	+
Shigella spp.	Environmental strain	-	-	-	-	-	-	+
Shigella spp.	MTCC 1457	-	-	-	-	-	-	+
Shigella spp.	Lab strain	-	-	-	-	-	-	+
Salmonella spp.	Clinical strain	-	-	+	-	-	-	-
Salmonella enterica	MTCC 733	-	-	+	-	-	-	-
Vibrio cholerae	MTCC 3906	-	-	-	-	-	+	-
Yersinia enterocolitica	MTCC 3100	-	-	-	+	-	-	-

Table 4.2. Primer sequences used in the PCR based assays

Name	Target gene	Primer	Sequence (5'-3')	Amplicon size	Accession No.	Primer position	Reference
Shigella spp.	Invasion Plasmid Antigen H	*IpaH-F* *IpaH-R*	CCTTGACCGCCTTTCCGATAC CAGCCACCCTCTGAGAGTACTC	611	M76444	376- 396 986- 965	(Fan *et al.*, 2008)
E. coli	Beta glucuronidase	*UidA-F* *UidA-R*	GTCACGCCGTATGTTATTG CCAAAGCCAGTAAAGTAGAAC	530	EF141499	37-55 966-986	(Babu *et al.*, 2013)
Vibrio cholerae	Outer membrane protein	*Omp W –F* *Omp W -R*	CACCAAGAAGGTGACTTTATTGTG GAACTTATAACCACCCGCG	588	MF100045	64-87 633-651	(Mehrabadi *et al.*, 2012)
Yersinia enterocolitica	Attachment Invasion Locus	*Ail –F* *Ail -R*	TACGCTGCGAGTGAAAGTAG GAATCGATACCCTGCACCAA	471	M29945	551-570 1002-1021	(Balakrishna *et al.*, 2010)
Pseudomonas aeruginosa	Gyrase B	*Gyr B –F* *Gyr B -R*	GGCGTGGGTGTGGAAGTC TGGTGGCGATCTTGAACTTCTT	190	AB039386	430-447 595-616	(Lee *et al.*, 2011)
Salmonella enterica	Invasion Plasmid Antigen B	*Ipa B –F* *Ipa B -R*	GGACTTTTTAAAAGCGGCGG GCCTCTCCCAGAGCCGTCTGG	315	U66877	723- 742 1037-1017	(Fan *et al.*, 2008)
Aeromonas hydrophila	Haemolysin gene	*Ahh1–F* *Ahh1 -R*	GCCGAGCGCCCAGAAGGTGAGTT GAGCGGCTGGATGCGGTTGT	130	20	961–983 1090–1071	(Wang *et al.*, 2003)

Table 4.3. PCR and multiplex PCR reaction mixture

Reagents	Final concentration		
	PCR	**Multiplex PCR Set 1**	**Multiplex PCR Set 2**
10X PCR buffer with $MgCl_2$	1 X	1 X	1 X
10 mMdNTP mix (2.5 mM each dNTP)	0.15 mM	0.15 mM	0.15 mM
Taq Polymerase enzyme (1 U/ µl)	1.5U	1.5U	1.5U
Primer (F), Primer (R) each of			
ahh1	0.2 µM	0.3 µM	
gyrB	0.2 µM	0.3 µM	
ipaB	0.2 µM	0.3 µM	
ail	0.2 µM		0.2 µM
uidA	0.2 µM	0.3 µM	
ompW	0.2 µM	0.2 µM	
ipaH	0.2 µM	0.2 µM	
Template DNA	1µl each	6µl	1 µl
Final Volume	50µl	50µl	50µl

Table 4.4. Temperature profile for PCR and multiplex PCR

Initial Denaturation	Denaturation	Annealing	Extension	Final extension
94 ^{o}C	94 ^{o}C	60 ^{o}C 52 ^{o}C (*Yersinia spp.)*	72 ^{o}C	72 ^{o}C
5min	30sec	1 min	1 min 40 cycles	10 min.

4.2.4. qPCR optimization

The composition of the qPCR reaction mixture is given in Table 4.5. A positive control containing known targets was always included along with no template control. Biorad, CFX96, Real time system was used to perform the qPCR experiment and Biorad CFX

manager software was used to analyze the results. And the cycling profile was: 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 60 °C for 1 min for all targets except *Yersinia spp* -52 °C for 1 min, with melt curve analysis between 65 -95 °C with an increase of 0.5 °C/Sec (Table 4.6).

Table 4.5. qPCR master mix composition

Reagents	**Final concentration qPCR**
2X qPCR master mix (SRL)	1 X
Primer (F), Primer (R) each of	
ahh1	0.5µM
gyrB	0.5µM
ipaB	0.5µM
ail	0.5µM
uidA	0.5µM
ompW	0.5µM
ipaH	0.5µM
Template DNA	1µl
Final Volume	10µl

Table 4.6. Temperature profile for qPCR

Initial Denaturation	**Denaturation**	**Annealing**	**Melt curve analysis**
94 °C	94 °C	60 °C 52 °C (*Yersinia spp.)*	65 °C 95 °C
5min	30sec	1 min- 40 cycles	0.5 °C/Sec.

4.2.5. Analytical specificity

Specificity has been evaluated with 35 bacterial strains that are strictly and distantly connected to the seven targets. All the isolates were cultured into the LB broth and extracted the DNA, used as a template in the PCR assay.

4.2.6. Analytical sensitivity and construction of the standard curve

To analyze sensitivity, 10^6cells/ml log-phase cultures of all reference bacterial pathogens were prepared and were 10-fold serially diluted in sterile saline to the final concentration from 10^6 to 10^0 cells/ml. 100 µl of each serial dilution was spiked into 100 ml autoclaved water and filtered through a 0.45 µm nitrocellulose membrane filter (Merck Millipore Ltd. Mumbai, India), which was then suspended in 1 ml of sterile phosphate-buffered saline (PBS) buffer and vigorously vortexes for 5 minutes. The membranes were removed and centrifuged the broth containing the isolated organisms at 15,000 g for 25 minutes. The total DNA extraction was performed using the modified Chen *et al.,* (2012) method. Standard curves were prepared for all the targets using reference strains, and the linear relationship between log DNA input, and Ct values were detected using qPCR assay. Using the formula, $E = 10^{-1/\text{slope}} - 1$, the amplification efficiency can be calculated (Park *et al.*, 2011).

4.2.7. Determination of limit of detection

4.2.7.1. Culture-based methods

The detection limit for conventional culture methods was calculated by the use of 10-fold serial dilution of target bacterial cells, ranging from 10^6 to 10^0 cells /ml, and the highest concentration of visible bacterial growth.

4.2.7.2. PCR based methods

The detection limit for PCR-based methods was calculated by the use of 10-fold serial dilution of the target DNA mixture, ranging from 10^6 to 10^0 cells/ml.

4.2.8. Cost-effectiveness of the selected methods

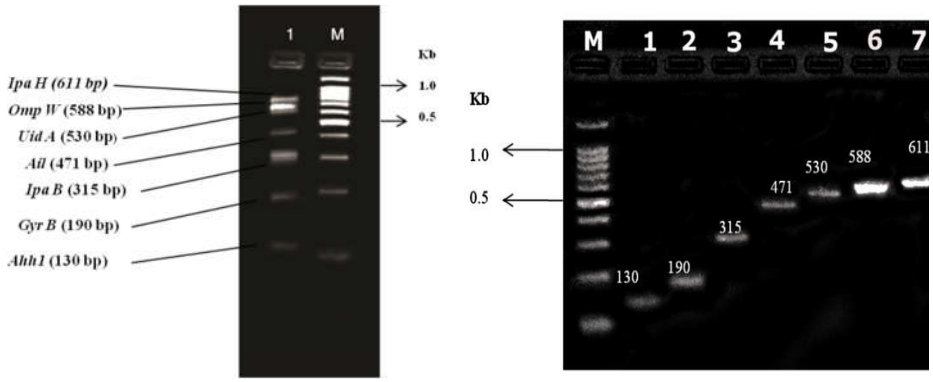
For each experiment, the cost of the components included expenses incurred for the culture media, experimental costs, reagents used for each sample evaluated (Table 4.9 and 4.10).

4.3. Results

4.3.1. Monoplex and multiplex PCR optimization

In this investigation, optimum conditions for monoplex PCR and afterward for multiplex PCR were initially standardized. It has been found that the PCR amplicons containing 130, 190, 315, 471, 530, 588 and 611 bp in bacterial strains belonging to *A. hydrophila, P. aeruginosa, S. enterica, Y. enterocolitica, E. coli, V. cholerae* and *Shigella spp.* In monoplex PCR, *Ahh1, GyrB, IpaB, Ail, UidA, OmpW* and *IpaH* gene primers were able to amplify the corresponding DNA.

No false positive or negative results have been found been found, confirming the exclusivity (Figure 4.1).



A) B)

Figure 4.1 Monoplex and multiplex PCR optimization. (A)Lane M; 100bp marker, lane 1; DNA mixture derived from *Aeromonas hydrophila (130bp), Pseudomonas aeruginosa (190bp), Salmonella enterica (315bp), Yersinia enterocolitica (471bp), E. coli (530bp), Vibrio cholerae (588bp)* and *Shigella spp (611bp)* was amplified using multiplex PCR. (B) Lane M; 100bp marker, Lane 1; *Aeromonas hydrophila (130bp), Lane 2; Pseudomonas aeruginosa (190bp), Lane 3; Salmonella enterica (315bp), Lane 4; Yersinia enterocolitica (471bp), Lane 5; E. coli (530bp), Lane 6; Vibrio cholerae (588bp)* and *Lane 7; Shigella spp (611bp)* was amplified using monoplex PCR.

4.3.2. Genus- and species-specificity of PCR primers

Oligonucleotide primers directed at *Ahh1* (130bp), *GyrB* (190bp), *IpaB* (315bp), *Ail* (471bp), *UidA* (530bp), *OmpW* (588bp) and *IpaH* (611bp) genes in bacterial strains belonging to *Aeromonas hydrophila, Pseudomonas aeruginosa, Salmonella enterica, Yersinia enterocolitica, E.coli, Vibrio cholerae,* and *Shigella spp.* respectively

(Table 4.2, Figure 4.1). To evaluate and verify the specificity of the primer pairs, DNA templates were prepared from a panel of 35 bacterial isolates, including control strains, and the PCR analysis showed 100 % specificities to corresponding organisms.

4.3.3. Determination of limit of detection

4.3.3.1. Culture based methods

The culture method detection limits for the seven target pathogens have been tested.10^2 cells/ml for *P. aeruginosa*, 10^3 cells/ ml for *S. enterica, E. coli* and *V. cholerae*. In the case of *Y. enterocolitica, A. hydrophila* and *Shigella spp.* it was found to be 10^4 cells/ml (Table 4.7). Due to the low detection sensitivity of the conventional culture methods, it needs high pathogen density in the sample to give positive results.

4.3.3.2. Monoplex and multiplex PCR

The detection sensitivity of the PCR based assays for the seven target pathogens was studied. As shown in Figure 4.2, a detection limit of multiplex PCR noted was 10^1 cells/ml for *P. aeruginosa* and *Shigella spp*. and 10^2 cells/ml for *A. hydrophila* and *V. cholerae*. In the case of *S. enterica, E. coli* and *Y. enterocolitica,* it was found to be 10^3 cells/ml (Figure 4.2 and Table 4.7). Despite that, our multiplex PCR system was somehow agreeable with monoplex PCR (Figure 4.3 and Table 4.7), in which a detection limit of 10^1 cells/ml was found for *Shigella spp. E. coli, V. cholerae* and *P. aeruginosa* and 10^2 cells/ ml for *A. hydrophila and Y. enterocolitica.*

The same 10^3 cells/ml were obtained for *S. enterica.* Most pathogens' infectious doses, including water-borne pathogens, were more than 10^3 contagious cells except *Shigella spp.*, which were 10-100 cells.

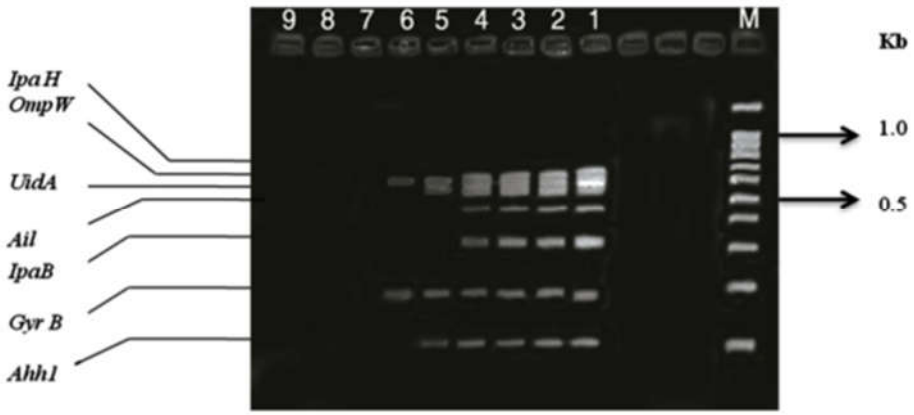
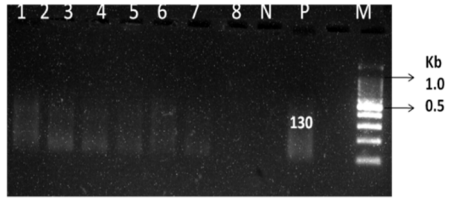
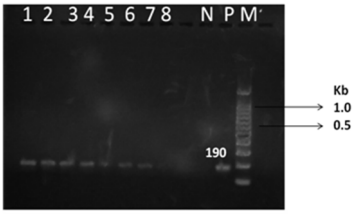


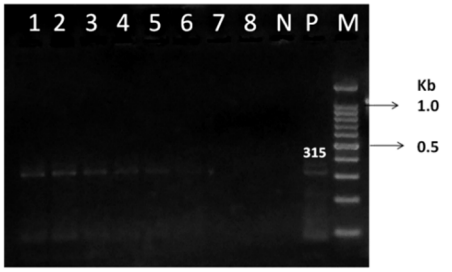
Figure 4.2 The sensitivity of multiplex PCR detection for seven different bacterial pathogens. Lane 1: 10^6 cells/ml; Lane 2: 10^5 cells/ml; Lane 3:10^4 cells/ml; Lane 4: 10^3 cells/ml; Lane 5: 10^2 cells/ml; Lane 6: 10^1 cells/ml; Lane 7:10^0 cells/ml; Lane M, DNA molecular size markers.



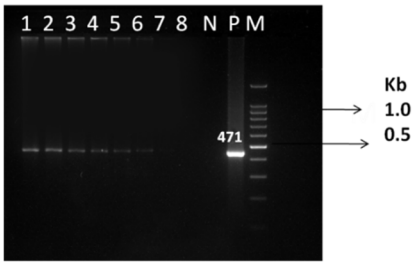
A) ***Aeromonas hydrophila- Ahh1-130bp***



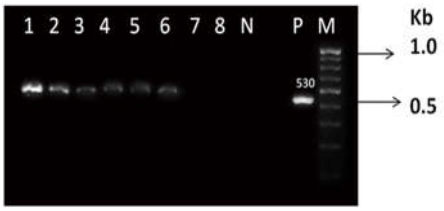
B) Pseudomonas aeruginosa- GyrB-190bp



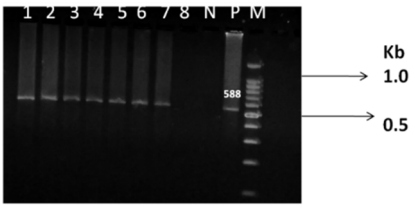
C) Salmonella enterica- IpaB- 315bp



D) Yersinia enterocolitica- Ail- 471bp



E) E.coli- UidA- 530bp



F) Vibrio cholerae- OmpW- 588bp

G Shigella spp. - IpaH-611bp

Figure 4.3 (A-G). The sensitivity of monoplex PCR detection for seven different bacterial pathogens. Lane 1: 10^7 Cells/ml; Lane 2: 10^6 Cells/ml; Lane 3:10^5 Cells/ml; Lane 4: 10^4 Cells/ml; Lane 5: 10^3 Cells/ml ; Lane 6: 10^2 Cells/ml ; Lane 7:10^1 Cells/ml ; Lane 8:10^0 Cells/ml ;Lane N, Negative control; Lane P, Positive control; Lane M, 100 bp DNA molecular size markers.

Table 4.7. Showing comparative analysis of detection limit of genes using selected methods

Organisms	Genes	Detection limit (cells/ml)			
		Culture	PCR	Multiplex PCR	qPCR
Aeromonas hydrophila	*ahh1*	10^4	10^2	10^2	1
Pseudomonas aeruginosa	*gyrB*	10^2	10^1	10^1	1
Yersinia enterocolitica	*ail*	10^4	10^2	10^3	1
Salmonella enterica	*ipaB*	10^3	10^3	10^3	1
Shigella spp.	*ipaH*	10^4	10^1	10^1	1
E. coli	*uidA*	10^3	10^1	10^3	1
Vibrio cholerae	*ompW*	10^3	10^1	10^2	1

4.3.4. qPCR

4.3.4.1. Standard Curve and Analytical Sensitivity

The standard curve ranged from 89 to 109 % amplification efficiencies of the target genes, and the analytical sensitivity was at one cell level for all targets. The overall regression lines are summarized in Table 4.8. Apart from the multiplex PCR sensitivity data, real time PCR the analytical sensitivity was at one cell (Table 4.7).

Table 4.8. Sensitivity and effectiveness of the quantitative real-time PCR.

Target and gene	Linear regression line	R^2	Efficiency (%)
Aeromonas hydrophila- ahh1	Y = −3.223lgX + 37.713	0.977	104
Pseudomonas aeruginosa- gyrB	Y = −3.122lgX + 34.975	0.966	109
Yersinia enterocolitica- ail	Y = −3.255lgX + 37.110	0.962	102.9
Salmonella enterica- ipaB	Y = −3.604lgX + 34.718	0.959	89
Shigella spp.- ipaH	Y = −3.466lgX + 36.237	0.977	94.3
E. coli- uidA	Y = −3.396lgX + 41.086	0.98	97
Vibrio cholerae- ompW	Y = -3.505lgX + 36.909	0.959	92.9

Table 4.9. Cost-effectiveness of the selected methods

Cost-effectiveness of selected methods/sample			
Culture	**PCR**	**Multiplex PCR**	**qPCR**
₹903	₹673	₹173	₹52

Table 4.10. Detailed table shows Cost effectiveness of the selected assays

Type of assay		Media	Price/100g (₹)	Cost /1000 samples
Culture	**1 stDay**			
	Most Probable Number method	Phenol Red Lactose broth	700	16, 800
	Total viable count	Nutrient agar	550	12, 100
	Selective Isolation	Membrane filtration	15481/100 filters	6, 19, 240
		Nutrient broth	420	273
		Alkaline peptone water	560	560
		Buffered peptone water	440	440
	2nd Day	Yersinia selective broth	687	687
		EMB agar	684	4, 788
		DCA agar	801	11, 214
		RV broth	742	1, 002
		TCBS agar	700	6, 300
		Aeromonas agar	822	3, 288
		King's B agar	1799	7, 196
	3rd Day	In case of 7 isolates/sample		
		PRLB	700	16800
		DCA agar	801	11214
		Gram staining	571/100ml	5, 710
		TSI media	761	3, 462
		Urease	740	777
		Nitrate	707	742
		Indole	497	521
		MR VP	490	980
		Citrate	449	561
		Catalase	40/400ml	10
		Oxidase	209	4, 180
		Carbohydrate fermentation	2894	14, 470
		MR VP	490	980
		Citrate	449	561
		Citrate	449	561
Total expenditure/1000sample				**₹9, 03, 779/-**

NB- Biochemical tests was done for every organisms isolated

Type of assay		Media	Price/100g (₹)	Cost /1000 samples (₹)
Molecular Methods	DNA EXTRACTION	Lysis buffer- 180 µl/sample	816/100g	0.24 g for 200ml- ₹ 2/0.25 g
		Tris Hcl	209/100g	1.6g for 200ml-₹ 2.3/1.6 g
		EDTA	2640/5g	0.05 g for 200ml-₹ 26/0.05g
		Lysozyme	1100	0.4 g for 200ml-₹ 4.4/0.4g
		SDS	180/500g	1.6 g for 200 ml-₹ 0.54/1.6g
		Nacl	661/10mg	20mg for 200ml-₹ 1322/20mg
		Proteinase K	598/1000 µl	200 µl -₹ 119
		Isopropanol	1219/100ml	100 ml -₹ 1219
		TE buffer	816/100g	0.24 g for 200ml- ₹ 2 /0.25 g
	Total	**₹ 2695 for all PCR, for each PCR, ₹ 2695/3= ₹ 898/-**		
	PCR	Master mix	2400/1ml	175 µl for 7 genes/sample, for1000 samples; 175000 µl =175ml=2400x175=Rs.4 20000
		Primers-F and R-7 Numbers	1000/each primer	1ml for 1000 samples-From each primer we can prepare 3.5ml working primer solution
				So₹ 1000 for PCR based 3 assays.Total for 49000 µl, ₹ 7000/-.
				In PCR, 14000 µl, so, ₹2000 for PCR
	AGE- 2 %	Agarose	2778/25g	2g in 100ml for 1 sample, 2000g for 1000samples, 80x25g=₹ 222, 240/-
		10X TBE	559/200ml	10ml for 1x preparation, in 90ml water/sample, so in 1000 samples, 10000ml, 50x559=₹ 27950/-
	Total expenditure/1000 samples		**₹ 6,73,088/-**	

Multiplex PCR	Master mix, Rs.2400/ml		50 µl for 2 sets/sample, for 1000 samples, 50000 µl=50ml=2400x50=₹ 120000/-
	Primers-F and R		1ml for 1000 samples- From each primer we can prepare 3.5ml working primer solution
			So₹ 1000 for PCR based 3 assays.Total for 49000 µl, ₹ 7000/-.
			In MPCR, 18000 µl, so, ₹ 2574 for MPCR
AGE-2 %	Agarose	2778/25g	2g in 100ml for 10 samples, 200g for 1000samples, 8x25g=₹ 7000=Rs.22224/-
	10X TBE	559/200ml	10ml for 1x preparation, in 90ml water/sample, so in 1000 samples, 10000ml, 50x559=₹ 27950/-
Total expenditure/1000samples		**₹ 1,73,646/-**	
qPCR	Master mix	₹.14591/1ml	Required 5 µl for 1 gene, 7genes requires 35 µl /sample, for 1000 sample 35x1000=35000 µl, 3.5ml 3.5mlx14591=₹ 51, 068/-
	Primers- F and R		1ml for 1000 samples- From each primer we can prepare 3.5ml working primer solution
			So₹ 1000 for PCR based 3 assays.Total for 49000 µl, ₹ 7000/-.
			In qPCR, 0.5 µl for each primer, so 7 µl for a sample, in 1000 samples, 7000µl=₹1000 for qPCR
No need of agarose run			
Total expenditure/1000 samples			**₹52, 966/-**

From the above results, it was clear that qPCR is a more robust, low-cost approach than conventional methods. Therefore, routine water analysis using these molecular methods helps to reduce assay costs and time requirements (Table 4.9 and Table 4.10).

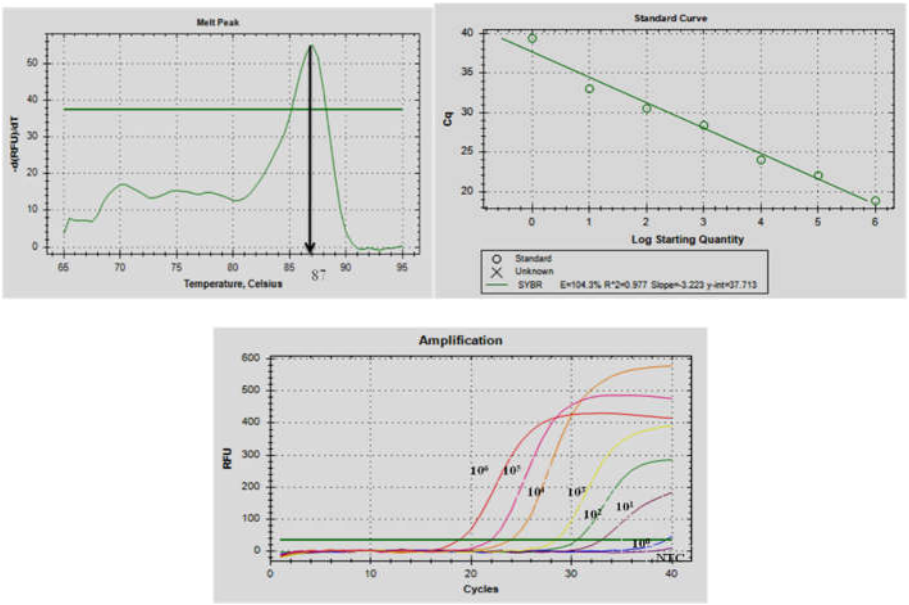
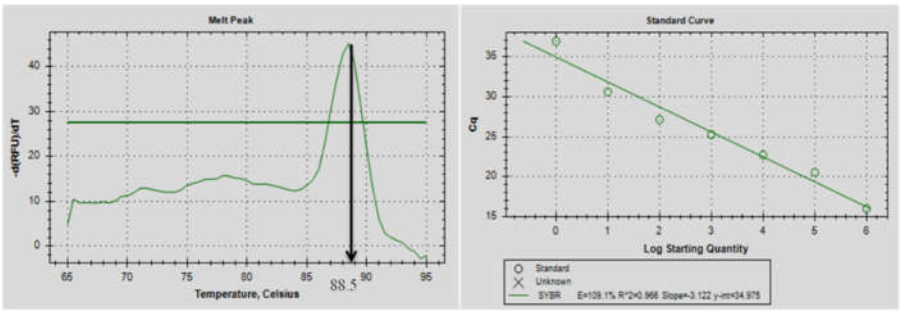
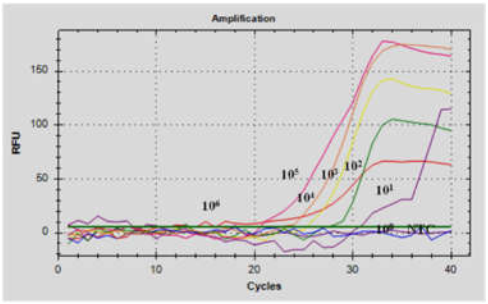
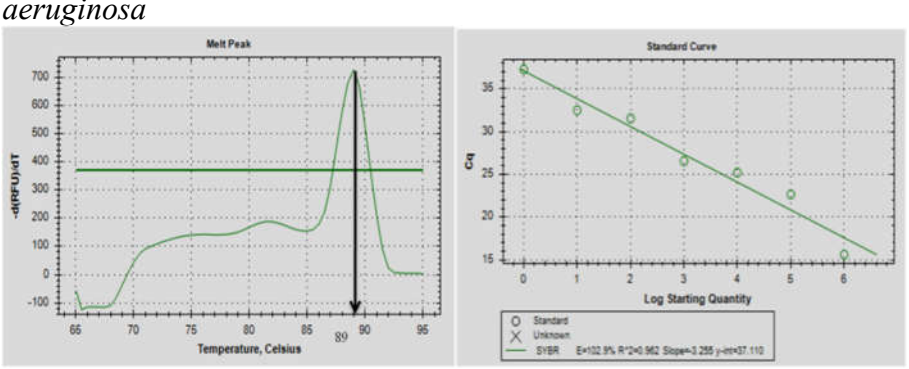


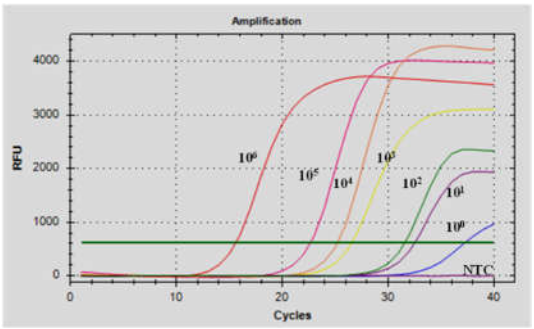
Figure 4.4 (1) Standard curve and melting temperature of *Aeromonas hydrophila*



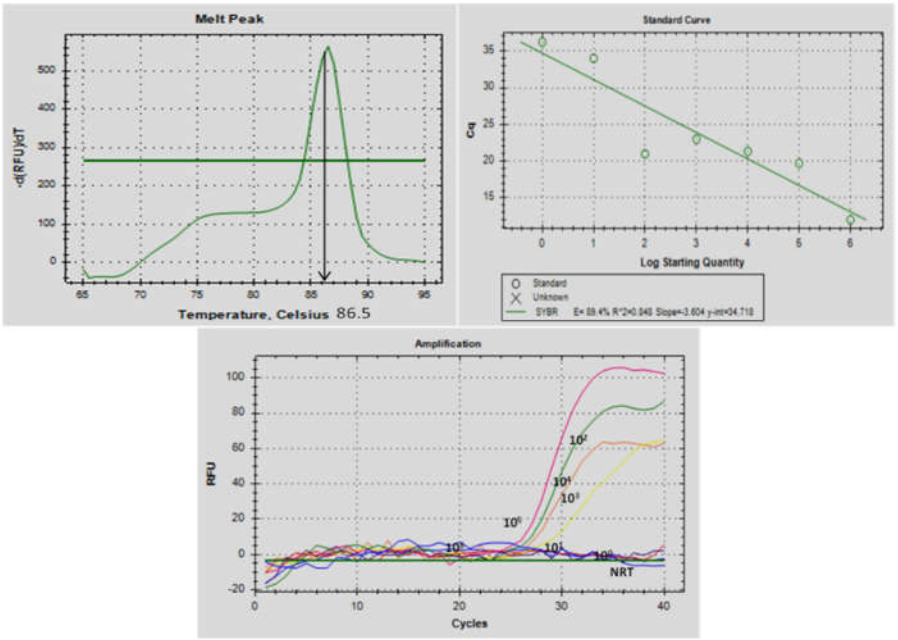


(2). Standard curve and melting temperature of *Pseudomonas aeruginosa*

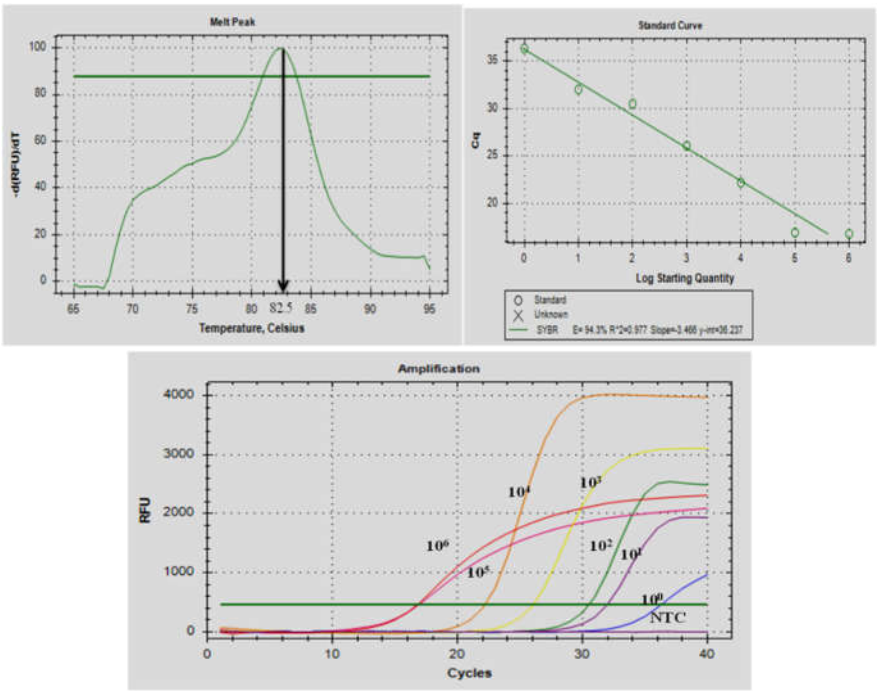




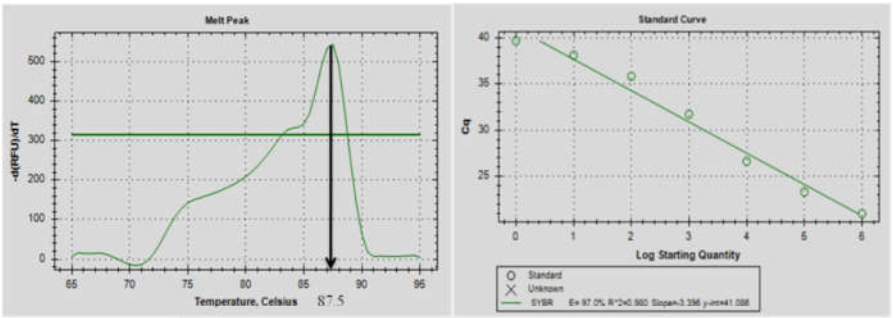
(3). Standard curve and melting temperature of *Yersinia enterocolitica*

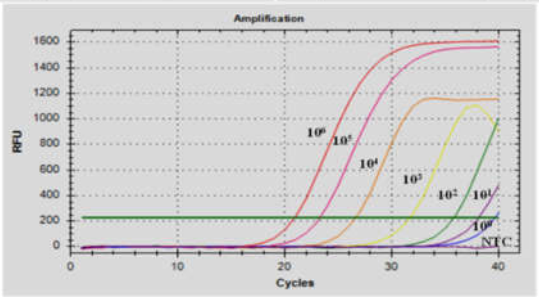


(4). Standard curve and melting temperature of *Salmonella enterica*

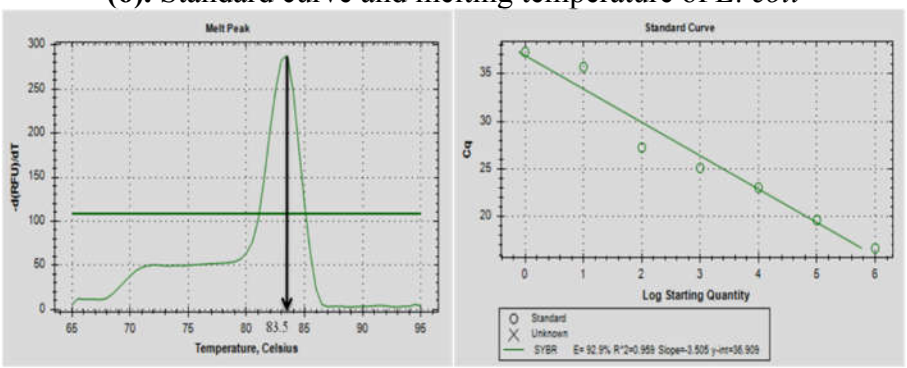


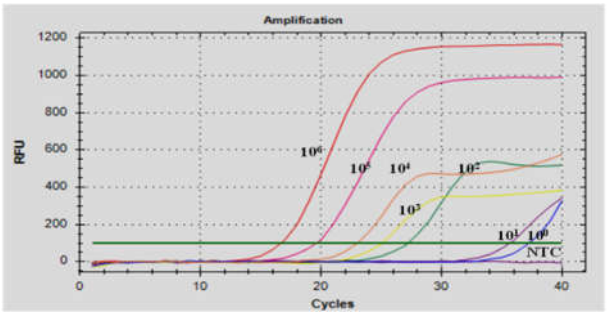
(5). Standard curve and melting temperature of *Shigella spp.*





(6). Standard curve and melting temperature of *E. coli*





(7). Standard curve and melting temperature of *Vibrio cholerae*

Figure 4.4 (1-7) Standard curve and melting temperature of different primers

4.4. Discussion

The main goal of this chapter was to develop a novel method for the co-detection and enumeration of several water-borne pathogens using multiplex PCR and qPCR assays. In water quality monitoring, fecal indicator bacteria are commonly used as surrogates for fecal pathogens. The ability to co-detect multiple water-borne pathogens is needed to move from the current status to a comprehensive health risk assessment based directly on specific pathogens. Nonetheless, multiplex PCR is ideal for this purpose. When quantitative PCR has been achieved, qPCR can identify and quantify pathogens now at concentrations as low as one target molecule per reaction.

We developed multiplex and real-time PCR assays for the simultaneous detection of seven water-borne pathogens, including *A. hydrophila, P. aeruginosa, S. enterica, Y. enterocolitica, E. coli, V. cholerae,* and *Shigella spp*., and found that these methods had high sensitivity and specificity. The assay's exclusivity was confirmed when all seven genes capable of amplifying the appropriate DNA provided no false positive or negative results. Furthermore, the detection sensitivity for the various PCR assays was within the range of 1 to 10^3 cells/ml, with 10^1 cells/ml for *P. aeruginosa* and *Shigella spp*., and 10^2 cells/ml for *A. hydrophila* and *V. cholerae*. In the cases of *S. enterica, E. coli*, and *Y. enterocolitica*, it was found to be 10^3 cells/ml, and our multiplex PCR results were in reasonable agreement with monoplex PCR. Castillo *et al.,* (2015) reviewed the detection sensitivity of various methods, including PCR, multiplex PCR, and real

time PCR. According to that review, PCR and Multiplex PCR falls in the range of detection sensitivity of 10 -100 cells/ml, and in the case of real time PCR it has got 1 cell/ml detection limit. According to our findings, the detection limit for PCR and multiplex PCR is 10 -1000 cells, and for qPCR it is 1 cell/ml. So the qPCR method we have developed has a sufficient detection limit (1 cell/ml) (Table 4.7).

Molecular based qPCR offers good sensitivity and it is considered an excellent analytical tool for detecting water-borne pathogenesis and helping to assess microbial risk. The detection limits for the various PCR based methods included were within the range of 10^3-1cells/ml. The infectious dose of most pathogens, including water-borne pathogens, was generally more than 10^3 contagious cells except for *Shigella spp*., which had an infectious dose of 10-100 cells. Other pathogens, including aquatic pathogens, had infectious doses of more than 10^3 contagious cells (Kong *et al.*, 2002; Fan *et al.*, 2008). Each species' infectious dosage differed, but the majority of water-borne bacteria cause disease when more than 10^3 contagious cells are consumed (Ramirez-Castillo *et al.*, 2015). As a result, the detection limit of our multiplex PCR assay was within the infectious dosage of the majority of enteric pathogens. Multiplex PCR is a cost-effective approach for rapid water sample screening. However, its sensitivity is slightly lower than monoplex PCR. Therefore, our multiplex PCR assay detection limit was within the infectious dose of most enteric pathogens.

The amplification efficiencies of standard curves range from 89-109 %, and analytical sensitivity was at one cell/ml level for real time PCR. These results are in good agreement with other studies which have shown that the analytical sensitivity was one copy/ µl for *E. coli O157: H7*, *L. monocytogenes/ivanovii, Shigella spp., β-Streptococcus hemolyticus, V. fluvialis* and *P. mirabilis* and ten copies/ µl for *Y. enterocolitica, V. parahaemolyticus, S. enterica, S. aureus, C. jejuni* and *E. faecalis* (Liu *et al.*, 2019). Table 4.7, 4.8, 4.9, and 4.10 compares the sensitivity and cost-effectiveness of various methods, concluding that qPCR is the best approach, with a detection limit of 1 cell/ml and a cost of around ₹ 52 per analyzed sample. Validation of the developed protocol using representative drinking water samples confirms that qPCR is the most cost-effective method for rapidly detecting water-borne pathogens in drinking water samples, compared to conventional culture methods and conventional PCR.

No false positive or negative results have been found in all seven genes, and we were able to amplify the corresponding DNA, confirming the exclusivity. Many researchers also succeed in developing multiplex PCR for the co-detection of other pathogens. Bo Li *et al.,* (2019) designed this type of assay to directly detect *E. coli, S. flexneri, S. enterica, C. jejuni, C. perfringens, L. pneumophila, L. monocytogenes* and *V. cholerae*. Giowanella *et al.,* (2015) detected the pathogenic strains of *E. coli* by targeting specific virulence genes for the detection of diarrhoeagenic *E. coli*, and also, the multidrug resistance of the isolates shows 51.04 % of the isolates shows MDR.

Kheiri *et al.,* (2016) developed two multiplex PCR assays for the simultaneous detection of six waterborne bacterial pathogens such as *E coli (uidA), Shigella (int), P. aeruginosa (gyrB), Salmonella (invA), V. choleare (ompW)* and coliforms (*lac Z)* with detection sensitivity of $3x10^2$-$3x10^3$ CFU respectively.

Aeromonas strains release several critical virulence factors for their pathogenicity. Most virulent strains secrete at least two types of hemolysin, one being channel forming aerolysin (*AerA*) and the other a non-channel forming hemolysin, *Ahh1*. The study conducted by Blaszk (2014) in River Nile strongly supported the suitability of the *Ahh1* gene as a valuable virulence marker for the identification of pathogenic *Aeromonas spp.* in water. According to RYC Kong *et al.*, (2002) it was found that the *IpaB* primers produce a common 315 bp amplicons to detect the most common *Salmonella* strains, this data is therefore consistent with our findings. One of the *Shigella* T3SS effectors, *IpaH* family proteins with E3 ubiquitin ligase activity and commonly retained among other bacterial pathogens are highly relevant as they facilitate bacterial survival by causing cell death and modulating the host's immune response. Studies using this *Ipa H* based PCR have been conducted in several Asian countries (Sethabutr *et al.*, 1994; Islam *et al.*, 1998; Dutta *et al.*, 2001).

Important food and water-borne bacterium *Y .enterocolitica* is known to cause many gastrointestinal problems. And the aquatic strains are highly heterogeneous, belonging to biotype 1A. Therefore, it is important to evaluate *Y. enterocolitica* as an emerging water-borne

pathogen further. Many PCR based studies were conducted to investigate the distribution of various virulence genes such as *ail, inv, yst, yadA, virF* and *yopT* in *Y. enterocolitica* (Fredriksson-Ahomaa *et al.*, 2011). The nucleotide sequence results showed that the *ompW* sequence is highly conserved in different biotypes or serotypes among *V.cholerae* strains (Nandi *et al.*, 2000). A study conducted by Anjana Sharma *et al.,* (2006) supports the idea that the cholera toxin is a useful tool for the detection of water-borne *V. cholerae*, which supports our results. The study uses species-specific *uidA* genes to detect *E. coli* by using beta-glucuronidase in aquatic samples, and the findings are supported by many researchers (Anklam *et al.*, 2012; Babu *et al.*, 2013).

The proposed method can be readily used in practice and can be successfully used for the routine monitoring of water quality. More research into the field of both multiplex PCR and qPCR is still necessary before obtaining a clear idea about all the water-borne pathogens, including bacteria, parasites, and viruses.

4.5. Conclusion

In conclusion, the developed multiplex PCR and qPCR assays represent a simple, rapid and powerful tool for the co-detection and enumeration of water-borne bacterial pathogens such as *A. hydrophila, P. aeruginosa, S. enterica, Y. enterocolitica, E. coli, V. cholerae,* and *Shigella spp* than conventional culture methods. And it is also helpful for the effective assessment of water treatment processes and helps to

provide more effective risk monitoring of possible threats to public health. Further studies can improve the detection limits and specificity of these methods.

CHAPTER 5

COMPARATIVE MOLECULAR EPIDEMIOLOGICAL STUDY OF qPCR AND MULTIPLEX PCR ASSAYS ON CULTURE-BASED METHODS FOR THE DETECTION OF WATERBORNE PATHOGENS

5.1. Introduction

The quality of drinking water from any source is continuously assessed to avoid water-borne outbreaks. Globally, water-borne diseases have been become a major public health problem, affecting half of the developing world's population. Water-borne diseases are not only a problem for developed nations, but they are also a significant challenge to developing countries (Deshmukh *et al.*, 2016). Conventional culture methods for detecting water-borne pathogens are time-consuming and laborious, and some microorganisms on bacteriological culture media are not culturable or the pathogen load is very less (Oliver, 2005).

Molecular methods are faster than traditional culture techniques, provide quick identification, and can potentially be adapted by health authorities to improve water quality monitoring. The main advantage of genetic markers for microbial detection is the relative stability of the genotype instead of the phenotype (Alhamlan *et al.*, 2015). Due to its versatility, specificity, and sensitivity, the polymerase chain reaction (PCR) is the most frequently used technique among molecular approaches. In this regard, a DNA fragment with a sequence shared only by specific species or strains can be selectively amplified. Chapter 4 presented a novel method for the co-detection and enumeration of several water-borne pathogens using multiplex PCR and quantitative real time PCR assays.

Our target pathogens, including *Aeromonas hydrophila, P. aeruginosa, S. enterica, Y. enterocolitica, E. coli, V. cholerae,* and *Shigella spp*. considered as water-borne, and outbreaks have been

reported due to the consumption of contaminated drinking water. In India, both well water and municipal water supply are the main drinking water sources in rural areas. Water can be a source of many pathogens through drinking water or irrigation, so preventing these diseases includes consistent water quality monitoring. But many factors can inhibit the pathogen monitoring, including viable but nonculturable bacteria, inhibitors from the soil, fecal inhibitors of nucleic acid-based assays, and low pathogen load in the sample. So the application of multiplex and real time quantitative PCR helps to reduce these problems associated with pathogen analysis.

Based on the molecular approaches developed in our study, this chapter's purpose is to apply these methods to the drinking water samples.

5.2. Materials and Methods

5.2.1. Collection of water samples

Sampling was conducted for one year, and 50 well water samples, 30 coastal water samples and 50 samples from the public water supply were collected. A 300 ml sample was collected in a sterile container under aseptic conditions from each sampling site, transported to the laboratory, and processed immediately.

5.2.2. Microbial enumeration in water samples

The multiple tube fermentation method was used to enumerate the total coliforms and total viable count by serially diluting the sample in sterile saline (Cappuccino & Sherman, 1996).

5.2.3. Selective isolation of target pathogens

Protocols for isolating and identifying *E. coli, S. enterica, Shigella spp., V. cholerae, P. aeruginosa, Y. enterocolitica* and *A. hydrophila* by using Bergey's Manual of determinative Microbiology (Bergey, D.H. & Holt., 1994)).

5.2.4. Prevalence of water-borne pathogens

Isolates from the samples were detected by using Bergey's Manual of determinative Microbiology (Bergey, D.H.& Holt., 1994).

5.2.5. Extraction of bacterial DNA from water samples

The sample was filtered through a 0.45µm nitrocellulose membrane filter and was then incubated in 2 ml of LB broth for 18-24 hours at 37 °C. After incubation, it was strongly vortexed for 2 min. The membranes were then removed, and the broth containing the separated microorganisms was centrifuged at 5, 000g for 5 min, after which the extraction was performed using the modified Chen *et al.,* (2012) method.

5.2.6. Application of multiplex PCR for the detection of target pathogens in water samples

The primes were selected from previous studies are based on their sensitivity and specificity. The reaction mixture consisted of 1 X PCR buffer (20 mM Tris hydrochloride pH 8.4, 50 mM KCl and 2.0 mM $MgCl_2$), 0.15 mM of each dNTP, 0.2 µM each of *IpaH, OmpW*,

and *Ail*, and 0.3 µM of *IpaB*, *UidA*, *Ahh1* and *GyrB* and 1.5 U of Hot Start Taq polymerase (Genei). A positive control containing known targets template DNA samples was always included, along with negative control with no template. The PCR was performed under the following conditions by the Bio-Rad, T^{100} thermal cycler: 94°C for 5 minutes, 40 cycles of 94 °C for 30 seconds, 60 °C for 1 minute for all targets except *Yersinia spp* (52 °C for 1 minute) extension at 72 °C for 1 minute) and the final extension at 72 °C for 10 minutes. The PCR amplicons were analyzed on 2 % agarose gel with 0.5 µg/mlethidium bromide and further visualized using the Bio-Rad Gel Doc 2000 documentation system.

5.2.7. Application of Quantitative Real-time PCR for the detection and the quantification of target genes in water Samples

The SYBR green real-time PCR assay was optimized using Biorad, CFX96, Real time system. A 10µL total volume reaction mixture consisted of 5 µL of Fluoro Green Premix EX Taq TM (SRL), 0.5µM of each primer 1 µL of DNA template, and water to volume along with positive control and non template control in each set and the assay was performed in duplicates. The PCR reaction was optimized to the conditions heat denaturation at 94 °C for 5 min followed by 40 cycles of heat denaturation at 94 °C for 30sec, primer annealing at 60 °C for 1 min (for *Y. enterocolitica* it was 52 °C for 1 min), and DNA extension at 72 °C for 1 min with fluorescence being measured during the extension phase. Melting curve analysis of the amplified DNA was

performed at a temperature range of 65 and 95 °C after amplification, with an increasing rate of 0.5 °C /s.

5.3. Results

A. Well water samples

A. 1. Conventional culture methods

A.1.1. The microbial enumeration in water samples.

The total coliform counts by MPN/100 ml in water samples collected ranged from 1100 MPN/100 ml to zero. The total viable count expressed as Log CFU/ml ranged from 6.67 to zero from these samples (Table 5.1).

Table 5.1. Mean values (log CFU/ml) of bacterial counts recorded on TVC at well water samples**.**

Sl. No	MPN index/100ml	95% confidence limits		Log CFU/ml
		Lower	Upper	
1	9	1	36	6.62±2
2	150	30	440	6.23±0.58
3	0	---	0.095	6.38±0.2
4	93	15	380	6±0.3
5	75	14	230	5.95±0.18
6	0	---	0.095	5±0.3
7	23	4	120	5.6±0.18
8	43	7	210	5
9	210	35	470	6.67±0.27
10	7	1	21	5±0.4
11	240	36	1300	5.85±0.3
12	0	---	0.095	5.48±0.12
13	75	14	230	6.04±0.21
14	0	---	0.095	5±0.4

15	0	---	0.095	5.4±0.12
16	0	---	0.095	5±0.4
17	43	7	210	5±0.38
18	1100	150	4800	5.9±0.28
19	23	4	120	5.3±0.3
20	240	36	1300	5.77±0.28
21	9	1	36	5.48±0.3
22	93	15	380	5.69±0.2
23	11	3	36	5±0.48
24	20	7	89	5.9±0.05
25	20	7	89	5.85±0.35
26	0	---	0.095	5
27	150	30	440	5.3±0.3
28	4	<0.5	20	5.78±0.13
29	0	---	0.095	5.48±0.4
30	0	---	0.095	5.69±0.2
31	28	10	150	5.6±0.6
32	14	3	37	5.3±0.18
33	23	4	120	5.48
34	0	---	0.095	5.48±0.2
35	4	<0.5	20	5
36	460	71	2400	5.6±0.2
37	11	3	36	5
38	240	36	1300	5±0.4
39	0	---	0.095	5.6±0.3
40	75	14	230	5
41	0	---	0.095	0
42	0	---	0.095	0
43	20	7	89	5.3±0.3
44	460	71	2400	5.6±0.25
45	28	10	150	5.68±0.2
46	240	36	1300	5.48±0.18
47	0	---	0.095	5
48	7	1	23	5.6±0.18
49	9	1	36	5.6±0.18
50	20	7	89	5.6±0.3

A. 1.2. Selective isolation of target pathogens

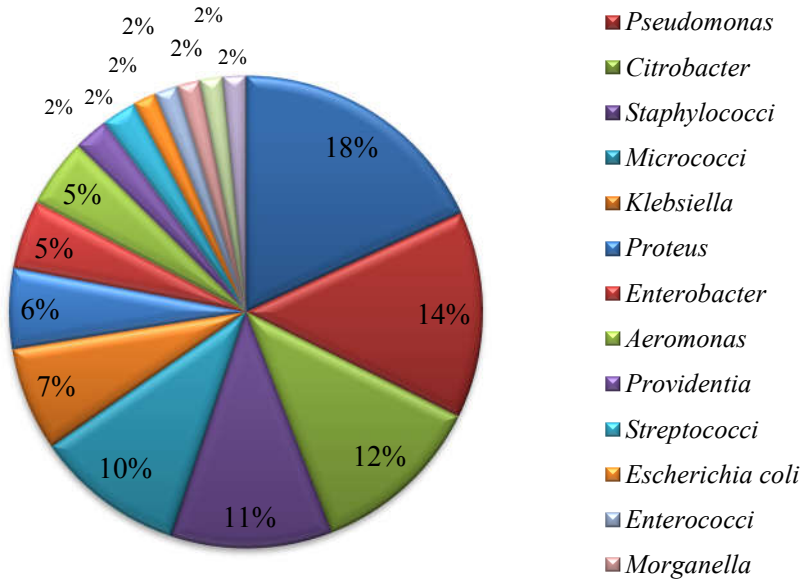
Only *E. coli, Pseudomonas spp.,* and *Aeromonas spp* were found to be culture positive, and others are culture negative. Therefore, the occurrence of target pathogens raises a concern about the raw drinking water usage.

Table 5.2. Selective isolation of target pathogens

Selective media	Pathogens	Interference	Positive samples
Eosin methylene blue (EMB)agar	*E. coli*	Large, blue-black colonies with a green metallic sheen	Positive
King's B agar	*Pseudomonas spp*	Greenish yellow colonies	Positive
Deoxy Cholate agar (DCA)	*Salmonella spp* *Shigella spp*	Colorless colonies with or without black color	Nil Nil
Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar	*V. cholerae*	Yellow colonies	Nil
Starch ampicillin agar (SAA)	*Aeromonas spp*	Produced honey color colonies surrounded by clear zone with Lugol's iodine	Positive
Yersinia selective agar (YSA)	*Yersinia spp*	Dark pink centered colonies	Nil

A.1.3. Prevalence of Isolated Organisms

Figure 5.1. Prevalence of isolated organisms in well water samples



The current study isolated and identified a total of 127 isolates from 50 well water samples collected from Malabar region of Kerala, among which 18.1 % (n=23) were *Bacillus spp*., 14.17 % (n=18) were *Pseudomonas aeruginosa*, 11.81 % (n=15) were *Citrobacter spp*., 11.02 % (n=14) were *Staphylococcus aureus* and 10.23 % (n=13) were *Micrococc*i. In addition to these isolates, a lower percentage of *Klebsiella spp*., (7.08 %, n=9), *Proteus spp*., (5.51 %, n=7), *Enterobacter spp*., and *Aeromonas spp*., (4.72 %, n=6), *Providencia spp*., and *Streptococcus spp*., (2.36 %, n=3), *E. coli*, *Enterococci, Morganella spp., Hafnia spp.,* and *Serratia spp.,* (1.57 %, n=2) were

also present. Their distribution and prevalence are given in Figure 5.1. The prevalence of these organisms in drinking water may lead to severe health problems.

A.2. Multiplex PCR

The multiplex PCR analysis of 50 well water samples has been conducted using the earlier approach mentioned. Due to reduced pathogen load requiring pre-enrichment, total bacterial DNA extracted from water samples was undertaken without culturing. As shown in Figure 5.3 (a) to (e), only four amplicons to the target genes *GyrB, Ahh1* and *UidA* were detected in different combinations. *IpaH* (*Shigella spp*.,), *OmpW* (*V. choleare*) or *Ail* (*Y. enterocolitica*), *IpaB* (*S. enterica*) have not been detected by multiplex PCR in the samples. *Ahh1* of *A. hydrophila* was identified in 24 samples; *Gyr B* positive *P. aeruginosa* was detected in 29 samples; followed by *E. coli* with *UidA* were detected in 3 samples. At the same time, no PCR products can be found in 15 samples. Finally, the PCR band intensities vary in all samples suggesting variations in the target cell densities. If this is valid, it has been found that there is no relation between the presence of the indicator organisms *E. coli* and other pathogens.

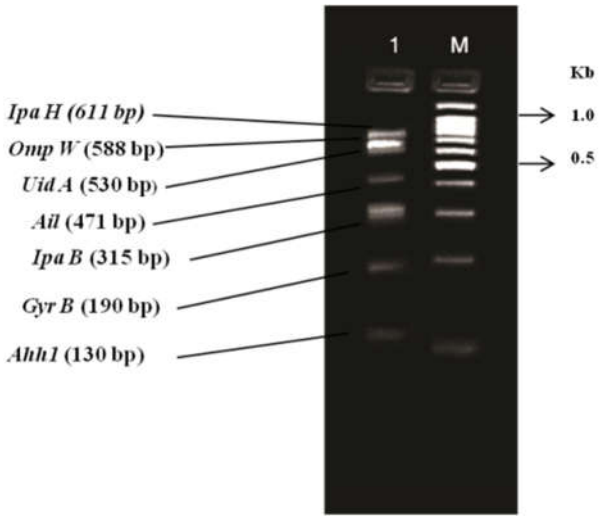


Figure 5.2. Standardised multiplex PCRLane M- 100bp DNA ladder; Lane P-Positive control

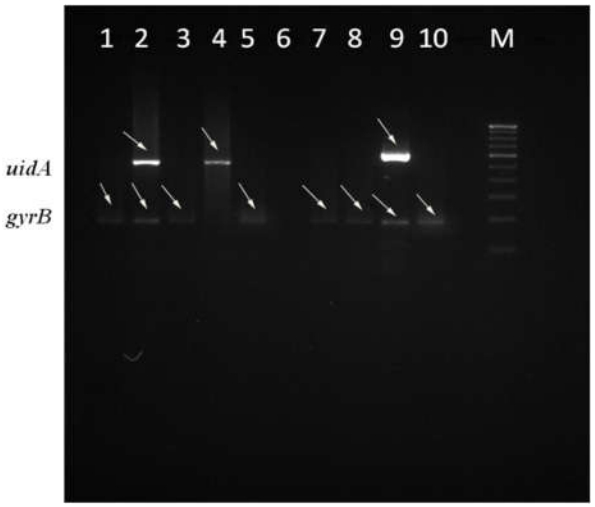


Figure 5.3. (a). Multiplex PCR of samples 1-10 (Lane 1-10); Lane M-Marker (100bp)

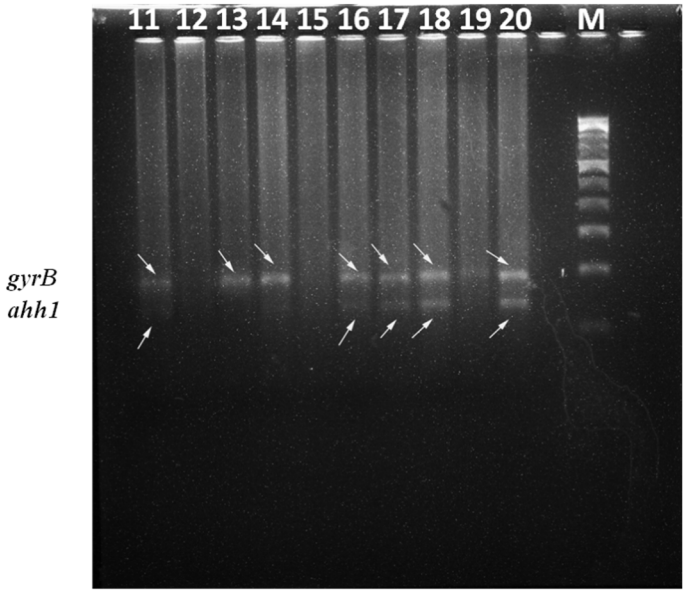


Figure 5.3. (b). Multiplex PCR of samples 11-20 (Lane 11-20); Lane M- Marker (100bp)

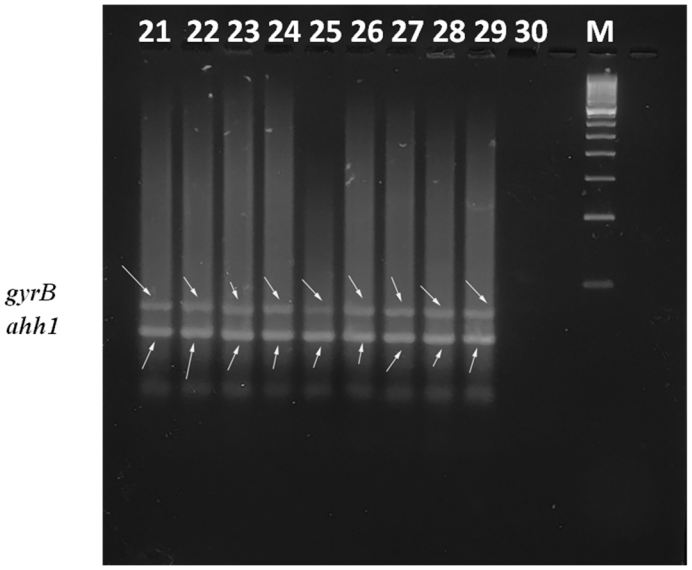


Figure 5.3. (c). Multiplex PCR of samples 21-30 (Lane 21-30); Lane M- Marker (200bp)

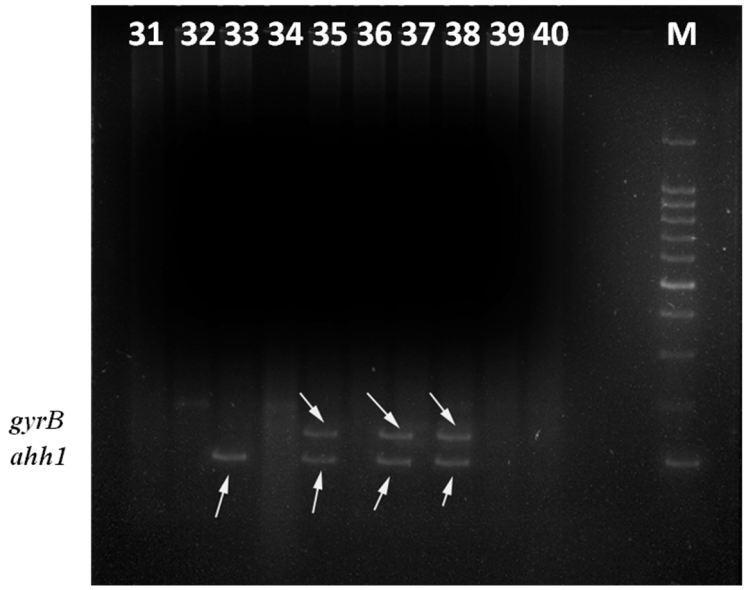


Figure 5.3. (d). Multiplex PCR of samples 31-40 (Lane 31-40); Lane M- Marker (100bp)

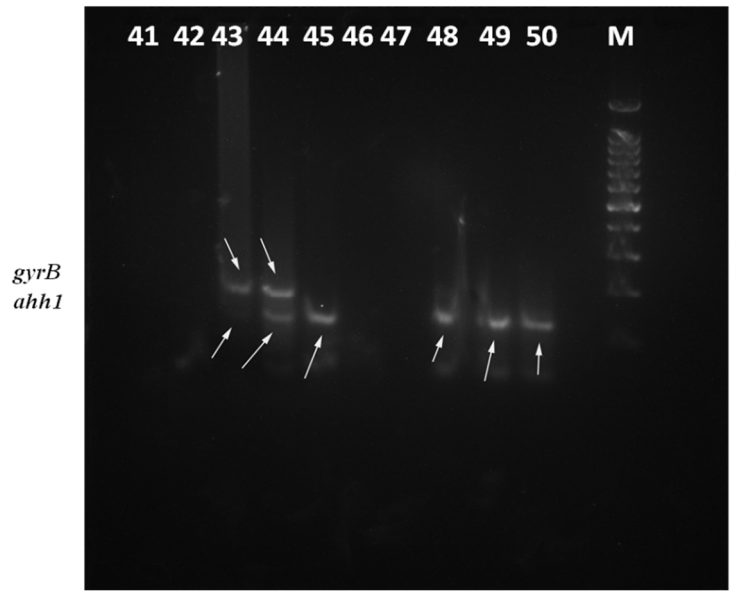


Figure 5.3. (e). Multiplex PCR of samples 41-50 (Lane 41-50); Lane M- Marker (100bp)

A. 3. Quantitative Real time PCR (qPCR)

The detection sensitivities were evaluated using standard strains, and a standard curve was constructed using 1×10^6- 1×10^0 cells/ml. Table 5.3 shows the data.

Table 5.3. Sensitivities of qPCR detected target genes

Threshold cycle							
Concentration	***A.hydrophila***	***Y.enterocolitica***	***P.aeruginosa***	***V.cholerae***	***Shigella spp.***	***S.enterica***	***E coli***
10^6	18.84	15.61	16.01	16.69	16.81	12.02	20.95
10^5	22.05	22.66	20.53	19.64	16.94	19.71	23.30
10^4	24.02	25.21	22.72	23.05	22.20	21.01	26.64
10^3	28.40	26.55	25.29	25.12	26.08	21.34	31.73
10^2	30.54	31.57	27.14	27.25	30.52	23.01	35.84
10^1	33.04	32.52	30.63	35.72	32.01	34.02	38.14
10^0	39.42	37.29	36.95	37.29	36.33	36.23	39.69

Quantitative analysis by real time PCR, by using this equation,

$$Q=10^{(Ct-b/m)}$$

Q=Quantity of DNA in the unknown sample

Ct=Ct value of the unknown sample

b=Intercept

m=Slope

A. 3.1. *Pseudomonas aeruginosa*

Quantitative analysis of *Pseudomonas aeruginosa* by real time PCR, by using this equation,

$$Y = -3.122 \text{ (Slope) } \lg X + 34.975 \text{ (Intercept)}$$

Table 5.4. Quantitative analysis of *Pseudomonas aeruginosa*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	16.64/17.14	$6.4\times10^5/1.6\times10^5$	26	25.80/26.82	$6.4\times10^2/3.2\times10^2$
2	22.50/20.62	$2.5\times10^4/2.1\times10^4$	27	17.33/17.54	$4.2\times10^5/0.5\times10^5$
3	15.00/ 15.65	$2\times10^6/0.71\times10^6$	28	24.36/24.30	$2.6\times10^3/0.08\times10^3$
4	19.50/19.47	$9.2\times10^4/0.18\times10^4$	29	27.96/22.49	$5.1\times10^3/6.9\times10^3$
5	27.94/22.16	$6.4\times10^3/8.9\times10^3$	30	ND	ND
6	22.22/22.51	$1.1\times10^4/0.16\times10^4$	31	ND	ND
7	22.0/21.08	$2.1\times10^4/0.97\times10^4$	32	32.60/32.16	6.89/1.6
8	21.00/20.94	$2.1\times10^4/0.97\times10^4$	33	36.60/30.87	11/14
9	19.80/18.79	$1.1\times10^5/0.54\times10^5$	34	ND	ND
10	19.47/18.11	$1.7\times10^5/1.1\times10^5$	35	23.57/22.33	$4.2\times10^4/2.5\times10^4$
11	19.31/19.89	$8.6\times10^4/2.5\times10^4$	36	ND	ND
12	20.91/19.86	$5.1\times10^4/2.6\times10^4$	37	24.10/25.08	$2.3\times10^3/1.1\times10^3$
13	16.95/16.86	$6.1\times10^5/0.29\times10^5$	38	30.49/30.68	$2.6\times10^1/0.3\times10^1$
14	19.57/19.60	$8.5\times10^4/0.13\times10^4$	39	ND	ND
15	32.32/36.93	3.7/4.8	40	32.14/32.16	8.3/0.08
16	18.57/18.32	$1.9\times10^5/0.26\times10^5$	41	27.30/24.16	$1.6\times10^3/1.9\times10^3$
17	16.94/17.13	$5.6\times10^5/0.55\times10^5$	42	26.21/27.75	$4.2/\times10^2 3.1\times10^2$
18	17.31 /17.50	$4.3\times10^5/0.42\times10^5$	43	26.82/24.20	$1.6\times10^4/1.7\times10^4$

19	ND	ND	44	23.57/22.33	$7.9\times10^3/4.8\times10^3$
20	16.96 /17.17	$5.5\times10^5/0.6\times10^5$	45	ND	ND
21	16.63 /17.72	$5.4\times10^5/2.9\times10^5$	46	ND	ND
22	17.29 /17.51	$4.3\times10^5/0.49\times10^5$	47	ND	ND
23	31.05/32.33	3.64/4.81	48	ND	ND
24	27.04/27.53	$2.9\times10^2/0.7\times10^2$	49	ND	ND
25	26.38/23.77	$2.2\times10^3/2.3\times10^3$	50	ND	ND

Positive control	15.06/15.29	$2.2\times10^6/0.3\times10^6$
Non template control-NTC	ND	ND

ND-Not Detected

A. 3.2. *Aeromonas hydrophila*

Quantitative analysis of *Aeromonas hydrophila,* by real time PCR, using this equation,

$$Y = -3.223 \text{ (Slope) } \lg X + 37.713 \text{(Intercept)}$$

Table 5.5. Quantitative analysis of *Aeromonas hydrophila*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	31.03/32.16	85.6/46.4	26	21.58/21.65	$9.9\times10^4/3.5\times10^4$
2	ND	ND	27	24.61/31.36	$5.9\times10^3/8.2\times10^3$
3	ND	ND	28	19.84/20.58	$2.8\times10^5/1.01\times10^5$
4	32.47/33.90	28.8/19.1	29	28.79/29.28	$5.02\times10^2/1.2\times10^2$
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	32.24/31.51	66.9/24.2	32	ND	ND

8	ND	ND	33	23.57/22.33	$4.2\times10^4/2.5\times10^4$
9	ND	ND	34	ND	ND
10	38.26/37.11	1.11/0.61	35	26.21/27.75	$2.5\times10^3/1.7\times10^3$
11	21.27/22.05	$9.9\times10^4/3.8\times10^4$	36	ND	ND
12	38.38/36.74	1.31/0.97	37	25.62/23.94	$1.2\times10^3/9.3\times10^3$
13	ND	ND	38	31.02/29.71	$2.12\times10^2/1.3\times10^2$
14	38.61/38.11	0.64/0.16	39	ND	ND
15	ND	ND	40	ND	ND
16	23.07/23.08	$3.5\times10^4/0.02\times10^4$	41	35.60/33.90	9.9/7.6
17	26.82/24.20	$8.9\times10^3/9.3\times10^3$	42	39.42/37.54	0.7/0.6
18	27.30/24.16	$8.9\times10^3/10.1\times10^3$	43	24.92/23.11	$2.2\times10^4/1.7\times10^4$
19	38.53/36.69	1.32/1.07	44	20.21/19.91	$3.1\times10^5/0.3\times10^5$
20	26.63/24.92	$6.03\times10^3/4.6\times10^3$	45	24.37/20.71	$1.0\times10^5/1.2\times10^5$
21	21.58/21.65	$9.9\times10^4/0.35\times10^4$	46	39.45/39.01	0.3/0.07
22	24.61/31.36	$5.9\times10^3/8.2\times10^3$	47	ND	ND
23	22.30/22.34	$5.9\times10^4/0.12\times10^4$	48	21.03/22.20	$1.1\times10^5/0.6\times10^5$
24	21.37/21.42	$1.2\times10^5/0.03\times10^5$	49	23.50/21.23	$7.8\times10^4/7.4\times10^4$
25	22.44/22.55	$5.3\times10^4/2.9\times10^4$	50	19.70/20.05	$3.5\times10^5/0.6\times10^5$

Positive control	16.3/18.39	$2.7\times10^6/2.4\times10^6$
Non template control-NTC	ND	ND

ND-Not Detected

A. 3.3. *E. coli*

Quantitative analysis of *E. coli* by real time PCR, by using this equation,

$$Y = -3.396 \text{ (Slope) } \lg X + 41.086 \text{ (Intercept)}$$

Table 5.6. Quantitative analysis of *E. coli*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	21.34/21.56	$6.1\times10^5/0.6\times10^5$	27	ND	ND
3	ND	ND	28	ND	ND
4	23.12/21.39	$4.1\times10^5/3.1\times10^5$	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	20.36/20.72	$1.1\times10^6/0.2\times10^6$	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	32.82/32.90	$2.71\times10^2/2.57\times10^2$	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	34.27/34.25	$1.01\times10^2/1.03\times10^2$
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	39.9/39.21	2.23/3.56
25	ND	ND	50	ND	ND

Positive control	19.50/19.54	$2.2\times10^6/0.04\times10^6$
Non template control-NTC	ND	ND

ND-Not Detected

A. 3.4. *Vibrio cholerae*

Quantitative analysis of *V. cholerae* by real time PCR by using this equation,

$$Y = -3.505 \text{ (Slope) } \lg X + 36.909 \text{ (Intercept)}$$

Table 5.7. Quantitative analysis of *V. cholerae*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	ND	ND
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control			18.81/19.67		$1.1 \times 10^5/0.5 \times 10^5$
Non template control-NTC			ND		ND

ND-Not Detected

A. 3.5. *Salmonella enterica*

Quantitative analysis of *Salmonella enterica* by real time PCR, by using this equation,

$$Y = -3.604 \text{ (Slope) } \lg X + 34.718 \text{ (Intercept)}$$

Table 5.8. Quantitative analysis of *Salmonella enterica*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	26.98/26.92	$1.43\times10^2/0.03\times10^2$	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	24.20/25.05	$6.6\times10^2/2.5\times10^2$	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	26.53/26.87	$1.7\times10^2/0.3\times10^2$	45	ND	ND
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control		23.81/23.98		$1.0\times10^3/0.07\times10^3$	
Non template control-NTC		ND		ND	

ND-Not Detected

A. 3.6. *Yersinia enterocolitica*

Quantitative analysis by real time PCR, by using this equation,

$$Y = -3.255 \text{ (Slope) } \lg X + 37.110 \text{ (Intercept)}$$

Table 5.9. Quantitative analysis of *Yersinia enterocolitica*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	39.31/38.66	0.3/0.1	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	ND	ND
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control			19.51/15.73		$1.9 \times 10^6/2.4 \times 10^6$
Non template control-NTC			ND		ND

ND-Not Detected

A. 3.7. Shigella *spp.*

Quantitative analysis by real time PCRby using this equation,

$$Y = -3.466 \text{ (Slope) } \lg X + 36.237 \text{ (Intercept)}$$

Table 5.10. Quantitative analysis of *Shigella spp*.

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	ND	ND
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control			15.50/15.45		$9.8 \times 10^5/0.2 \times 10^5$
Non template control-NTC			ND		ND

ND-Not Detected

Table 5.11. Comparison of qPCR, multiplex PCR, and conventional methods- well water samples

Sl. No	*Pseudomonas aeruginosa*			*Aeromonas hydrophila*			*E. coli*			*Salmonella enterica*			*Vibrio cholerae*			*Yersinia enterocolitica*			*Shigella ssp.*		
	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT
1	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
4	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
5	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
8	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
10	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
16	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

18	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	+	+	+	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
21	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

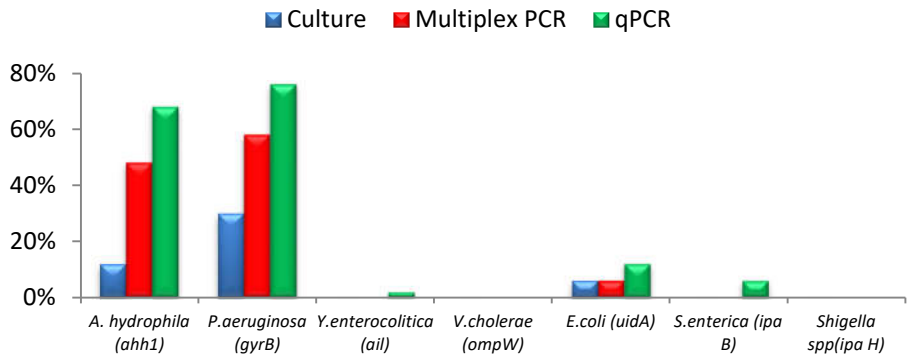
40	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
43	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
46	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
50	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	**15**	**29**	**38**	6	24	34	3	3	6	0	0	3	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**

C= Culture, M=Multiplex PCR, qRT=Quantitative real time PCR.

Table 5.12. Detection of target pathogens using culture, multiplex PCR and qPCR

Techniques	% Detection of target pathogens						
	A. hydrophila (ahh1)	***P. aeruginosa (gyrB)***	***Y. enterocolitica (ail)***	***V. cholerae (ompW)***	***E. coli (uidA)***	***S. enterica (ipaB)***	***Shigella spp (ipaH)***
Culture	12% (6/50)	30% (15/50)	0 (0/50)	0 (0/50)	6% (3/50)	0 (0/50)	0 (0/50)
Multiplex PCR	48% (24/50)	58% (29/50)	0 (0/50)	0 (0/50)	6% (3/50)	0 (0/50)	0 (0/50)
qPCR	68% (34/50)	76% (38/50)	2% (1/50)	0 (0/50)	12% (6/50)	6% (3/50)	0 (0/50)

Figure 5.4. Detection of target pathogens using culture, multiplex PCR and qPCR



The *P. aeruginosa* with the *GyrB* gene was the most prevalent in all the samples, and it was found to be 30 %, 58 % and 76 % by culturing, multiplex PCR, and real time PCR assays, respectively. They were followed by *A. hydrophila* carrying the *Ahh1* gene with 12 %, 48 %, and 68 %, respectively. The indicator organisms *E. coli* were 6 % culture-positive and in multiplex PCR and

12 % in qPCR. *Y. enterocolitica* has the *Ail* gene, *OmpW* gene carried by *V. cholerae*, *S. enterica* with *Ipa B* and *Shigella spp.* having *IpaH* were not detected by culture methods throughout the sampling period. When coming to *S. enterica* no multiplex PCR was found to be positive, and the qPCR data showed that 6 % were positive for *IpaB* genes. The results show that no *V. cholerae* were found to be detected by both multiplex PCR and qPCR. Neither *Yersinia spp*. nor *Shigella spp*. was detected in multiplex PCR, but 2 % positive results were shown in qPCR in the case of *Yersinia spp*. However, *Shigella spp*. was not detected in all the samples by all three methods (Table 5.12, Figure 5.4).

B. Public water supply

B.1. Conventional culture methods

B. 1.1. Microbial enumeration in water samples

The total coliform counts by MPN/100 ml in water samples collected ranged from 93 MPN/100 ml to zero. The total viable count expressed as Log CFU/ml ranged from 4.9 to zero from these samples.

Table 5.13. Mean values (log CFU/ml) of bacterial counts recorded on TVC at public water samples.

Sl. No	MPN index/100ml	95% confidence limits		Log CFU/ml
		Lower	Upper	
1	0	-	0.095	4
2	0	-	0.095	4±0.3
3	11	3	36	0
4	4	<0.05	20	0
5	0	-	0.095	0
6	0	-	0.095	4
7	9	1	36	0
8	93	15	380	4±0.3
9	0	-	0.095	0
10	23	4	120	4
11	0	-	0.095	0
12	0	-	0.095	4.3±0.1
13	20	7	89	4.90±0.25
14	0	-	0.095	0
15	4	<0.05	20	4.69
16	7	1	21	4.30±0.17
17	93	15	380	4.3
18	0	-	0.095	4.60±0.2
19	0	-	0.095	4.47±0.17
20	0	-	0.095	4
21	4	<0.05	20	4.60±0.3
22	23	4	120	0
23	0	-	0.095	0
24	0	-	0.095	0
25	43	7	210	4.77±0.17
26	9	1	36	4
27	0	-	0.095	0
28	7	1	21	4.3±0.3
29	75	14	230	4.9±0.1
30	0	-	0.095	4.6

31	0	-	0.095	4
32	0	-	0.095	0
33	4	<0.05	20	4.47±0.2
34	0	-	0.095	4.84±15
35	14	3	37	4.60±0.13
36	0	-	0.095	0
37	0	-	0.095	0
38	0	-	0.095	4
39	0	-	0.095	4±0.6
40	11	3	36	4
41	7	1	21	0
42	75	14	230	4.60±0.17
43	0	-	0.095	0
44	0	-	0.095	0
45	20	7	89	4.60±0.6
46	0	-	0.095	4
47	0	-	0.095	4
48	28	10	150	4.604.60± 0.31
49	0	-	0.095	4.30±0.3
50	9	1	36	4

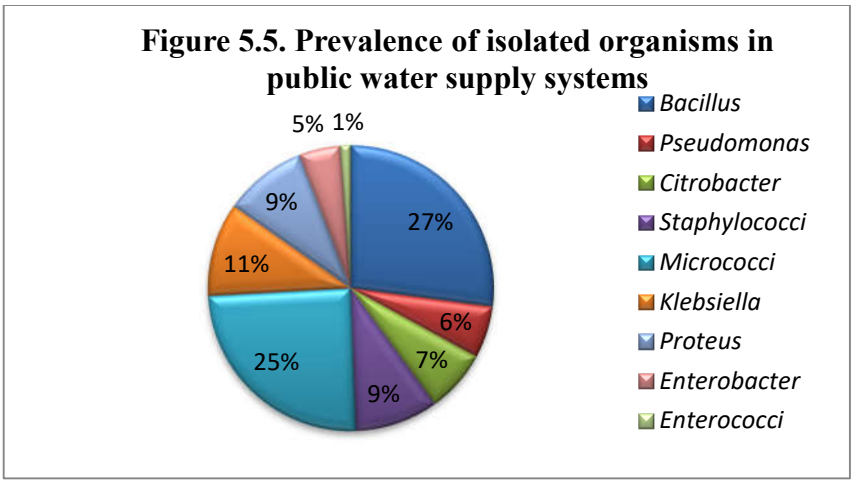
B. 1.2. Selective isolation of target pathogens

Only *Pseudomonas spp* was found to be culture positive.

Table 5.14. Selective isolation of target pathogens

Selective media	Pathogens	Interference	Positive samples
Eosin methylene blue (EMB)agar	*E. coli*	Large, blue-black colonies with a green metallic sheen	Nil
King's B agar	*Pseudomonas spp*	Greenish yellow colonies	Positive
Deoxy Cholate agar (DCA)	*Salmonella spp* *Shigella spp*	Colorless colonies with or without black color	Nil Nil
Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar	*V. cholerae*	Yellow colonies	Nil
Starch ampicillin agar (SAA)	*Aeromonas spp*	Produced honey color colonies surrounded by clear zonewithLugol's iodine	Nil
Yersinia selective agar (YSA)	*Yersinia spp*	Dark pink centered colonies	Nil

B. 1.3. Prevalence of Isolated Organisms



The current study isolated and identified a total of 85 isolates from 50 public water supply systems, among which 27.05 % (n=23) were found to be *Bacillus spp*., 24.7 % (n=21) was *Micrococci.,* 10.58 % (n=9) were found to be *Klebsiella spp*., 9.4 % (n=8) were *Staphylococcus aureus* and *Proteus spp*. In addition to these isolates, a lower percentage of *Citrobacter spp*., (7.05 %, n=6), *Pseudomonas spp*., (5.88 %, n=5), *Enterobacter spp.,* (4.7 %, n=4) and *Enterococci* (1.17 %, n=1) (Figure 5.5). No *E. coli* was isolated during the study.

B. 2. Multiplex PCR

As shown in Figure. 5.6 *P. aeruginosa* carrying *GyrB* was detected in different combinations of 8 samples and one *A. hydrophila* with *ahh1* gene was detected in a single sample. No other target genes were detected by multiplex PCR in the samples. According to the pathogen concentration, the band intensities may vary with samples. Neither indicator organism, *E. coli* nor other pathogens were detected

in these samples that may be due to proper treatment of the samples (Figure 5.6 a-e)

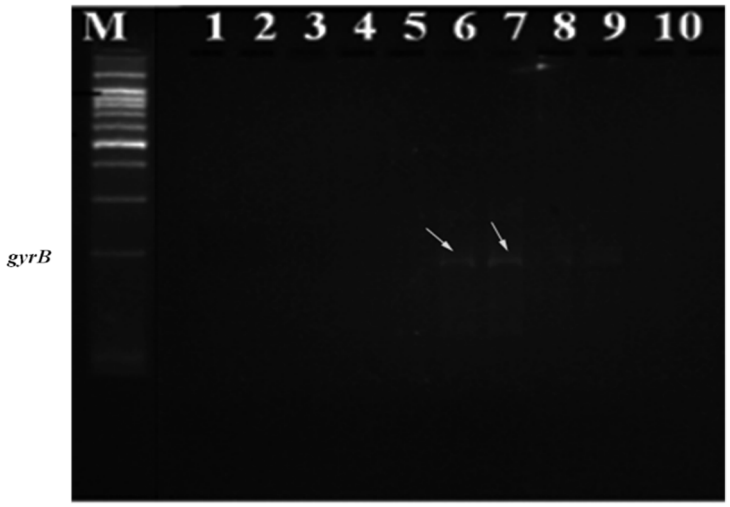


Figure 5.6. (a). Multiplex PCR of samples 1-10- public water supply systems (Lane 1-10) ;Lane M- Marker (100bp)

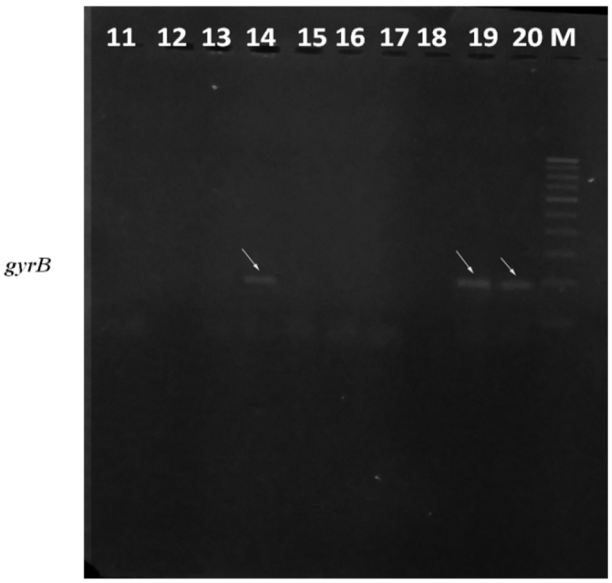


Figure 5.6. (b). Multiplex PCR of samples 11-20- public water supply systems (Lane 11-20) ;Lane M- Marker (100bp)

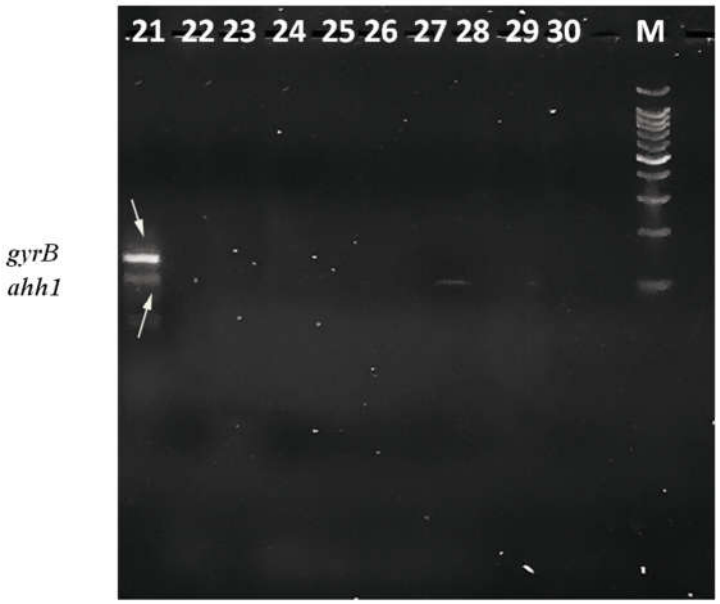


Figure 5.6. (c). Multiplex PCR of sample 21-30 public water supply systems (Lane 21-30) ;Lane M- Marker (100bp)

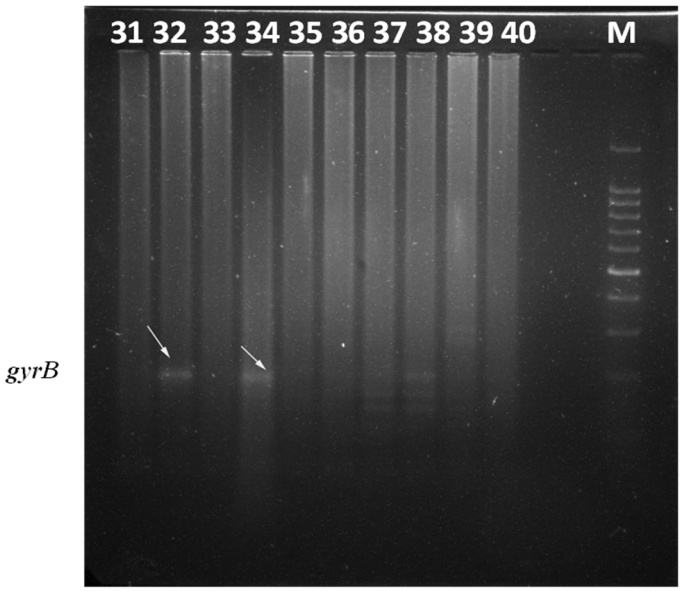


Figure 5.6. (d). Multiplex PCR of sample 31-40 public water supply systems (Lane 31-40) ;Lane M- Marker (100bp)

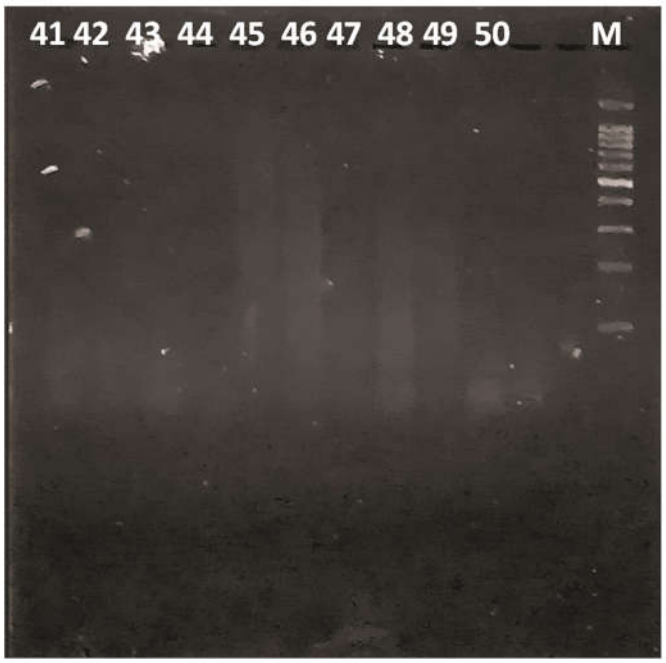


Figure 5.6. (e). Multiplex PCR of sample 41-50 public water supply systems (Lane 31-40) ;Lane M- Marker (100bp)

Quantitative analysis by real time PCR, by using this equation,

$$Q=10^{(Ct-b/m)}$$

Q=Quantity of DNA in the unknown sample
Ct=Ct value of the unknown sample
b=Intercept
m=Slope

B. 3.1. *Pseudomonas aeruginosa*

Quantitative analysis of *Pseudomonas aeruginosa* by real time PCR, by using this equation,

$$Y = -3.122 \text{ (Slope) } \lg X + 34.975 \text{ (Intercept)}$$

Table 5.15. Quantitative analysis of *Pseudomonas aeruginosa*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	29.65/30.09	70.6/47.9	31	ND	ND
7	29.98/28.45	81.4/58.8	32	28.82/29.36	78.3/21.8
8	ND	ND	33	ND	ND
9	ND	ND	34	29.21/29.20	70.5/0.4
10	30.29/29.78	38.9/10.2	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	30.07/29.54	46.2/12.6	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	24.97/23.05	$4.1\times10^3/3.5\times10^3$	44	ND	ND
20	24.55/25.05	$1.9\times10^3/0.5\times10^3$	45	ND	ND
21	22.70/23.13	$7.4\times10^3/1.6\times10^3$	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control		23.16/22.38		$8.5\times10^3/3.3\times10^3$	
Non template control-NTC		ND		ND	

ND-Not Detected

B. 3.2. *Aeromonas hydrophila*

Quantitative analysis of *Aeromonas hydrophila,* by real time PCR, using this equation,

$$Y = -3.223 \text{ (Slope) } \lg X + 37.713 \text{(Intercept)}$$

Table 5.16. Quantitative analysis of *Aeromonas hydrophila*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	ND	ND
21	24.38/23.14	$2.3\times10^4/1.4\times10^4$	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control			15.82/15.92	$6\times10^6/ 0.3\times10^6$	
Non template control-NTC			ND	ND	

ND-Not Detected

B. 3.3. *E. coli*

Quantitative analysis of *E. coli* by real time PCR, by using this equation,

$$Y = -3.396 \text{ (Slope) } \lg X + 41.086 \text{ (Intercept)}$$

Table 5.17. Quantitative analysis of *E. coli*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	ND	ND
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control			26.53/27.34	$1.5 \times 10^4/0.5 \times 10^4$	
Non template control-NTC			ND	ND	

ND-Not Detected

B. 3.4. *Vibrio cholerae*

Quantitative analysis of *V. cholerae* by real time PCR by using this equation,

$$Y = -3.505 \text{ (Slope) } \lg X + 36.909 \text{ (Intercept)}$$

Table 5.18. Quantitative analysis of *V. cholerae*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	ND	ND
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control			20.80/20.17		$4.9 \times 10^4/1.4 \times 10^4$
Non template control-NTC			ND		ND

ND-Not Detected

B. 3.5. *Salmonella enterica*

Quantitative analysis of *Salmonella enterica* by real time PCR, by using this equation,

$$Y = -3.604 \text{ (Slope) } \lg X + 34.718 \text{ (Intercept)}$$

Table 5.19. Quantitative analysis of *Salmonella enterica*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	ND	ND
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control			26.74/26.53	$1.8 \times 10^2/0.2 \times 10^2$	
Non template control-NTC			ND	ND	

ND-Not Detected

B. 3.6. *Yersinia enterocolitica*

Quantitative analysis by real time PCR, by using this equation,

$$Y = -3.255 \text{ (Slope) } \lg X + 37.110 \text{ (Intercept)}$$

Table 5.20. Quantitative analysis of *Yersinia enterocolitica*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	ND	ND
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control			24.93/25.53		$4.6 \times 10^3/1.4 \times 10^3$
Non template control-NTC			ND		ND

ND-Not Detected

B. 3.7. *Shigella spp.*

Quantitative analysis by real time PCR by using this equation,

$$Y = -3.466 \text{ (Slope) } \lg X + 36.237 \text{ (Intercept)}$$

Table 5.21. Quantitative analysis of *Shigella spp*.

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	26	ND	ND
2	ND	ND	27	ND	ND
3	ND	ND	28	ND	ND
4	ND	ND	29	ND	ND
5	ND	ND	30	ND	ND
6	ND	ND	31	ND	ND
7	ND	ND	32	ND	ND
8	ND	ND	33	ND	ND
9	ND	ND	34	ND	ND
10	ND	ND	35	ND	ND
11	ND	ND	36	ND	ND
12	ND	ND	37	ND	ND
13	ND	ND	38	ND	ND
14	ND	ND	39	ND	ND
15	ND	ND	40	ND	ND
16	ND	ND	41	ND	ND
17	ND	ND	42	ND	ND
18	ND	ND	43	ND	ND
19	ND	ND	44	ND	ND
20	ND	ND	45	ND	ND
21	ND	ND	46	ND	ND
22	ND	ND	47	ND	ND
23	ND	ND	48	ND	ND
24	ND	ND	49	ND	ND
25	ND	ND	50	ND	ND
Positive control			16.63/16.73		$4.4\times10^5/0.2\times10^5$
Non template control-NTC			ND		ND

ND-Not Detected

Table 5.22. Comparison of qPCR, multiplex PCR, and conventional methods- public water supply

Sl. No	*Pseudomonas aeruginosa*			*Aeromonas hydrophila*			*E. coli*			*Salmonella enterica*			*Vibrio cholerae*			*Yersinia enterocolitica*			*Shigella ssp.*		
	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

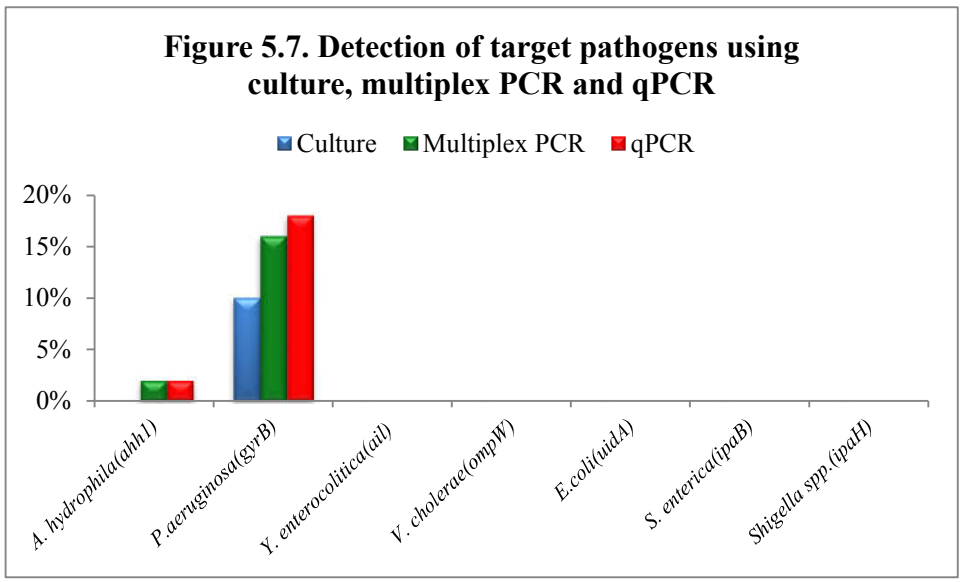
19	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	**5**	**8**	**9**	**0**	1	1	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

C= Culture, M=Multiplex PCR, qRT=Quantitative real time PCR.

Table 5.23. Detection of target pathogens using culture, multiplex PCR and qPCR

Techniques	% Detection of target pathogens						
	A. hydrophila (ahh1)	***P. aeruginosa (gyrB)***	***Y. enterocolitica (ail)***	***V. cholerae (ompW)***	***E. coli (uidA)***	***S. enterica (ipaB)***	***Shigella spp (ipaH)***
Culture	0% (0/50)	10% (5/50)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)
Multiplex PCR	2% (1/50)	16% (8/50)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)
qPCR	2% (1/50)	18% (9/50)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)



The *P. aeruginosa* with the *GyrB* gene was found in 10%, 16%, and 18 % by culture, multiplex PCR, and qPCR assays followed by *A. hydrophila* carrying the *Ahh1* gene with 2 % by both multiplex and qPCR assays and not detected by culture methods. The indicator organisms *E. coli*, *Y. enterocolitica*, *V. cholerae, S. enterica*,

and *Shigella spp.* were not seen by any of these methods (Table 5.23, Figure 5.7).

C. Coastal water

C. 1. Conventional culture methods

C. 1.1. Microbial enumeration in water samples

The total coliform counts by MPN/100 ml in water samples collected ranged from 1100 MPN/100 ml to zero. The total viable count expressed as Log CFU/ml ranged from 6.63 to zero from these samples.

Table 5.24. Mean values (log CFU/ml) of bacterial counts recorded on TVC at coastal water samples.

Sl. No	MPN indeX/100ml	95% confidence limits		Log CFU/ml
		Lower	Upper	
1	240	0.42	10	5.56±0.11
2	0	---	0.095	4±0.60
3	75	0.17	2	5.47±0.13
4	0	---	0.095	4.3±0.70
5	0	---	0.095	5.11±0.16
6	0	---	0.095	4
7	43	0.09	1.8	5.36±0.06
8	1100	1.8	41	5.56±0.04
9	23	0.05	0.94	4.60±0.13
10	240	0.42	10	5.50±0.03
11	1100	1.8	41	5.97±0.02
12	460	0.09	20	6.53±0.07
13	93	0.18	4.2	6.49±0.04

14	93	0.18	4.2	6.55±0.04
15	23	0.05	0.94	6.62±0.. 02
16	150	0.37	4.2	5.11±0.1
17	1100	1.8	41	0
18	460	0.9	20	6.32±0.02
19	1100	1.8	41	4.90±0.17
20	460	0.09	20	5.43±0.17
21	1100	1.8	41	4.30±0.5
22	>1100	4.2	---	5.04±0.2
23	23	0.05	0.94	0
24	75	0.17	2	4.3
25	>1100	4.2	---	4.95±0.2
26	93	0.18	4.2	4
27	>1100	4.2	---	5.36±0.6
28	93	0.18	4.2	0
29	23	0.05	0.94	0
30	9	0.01	0.38	0

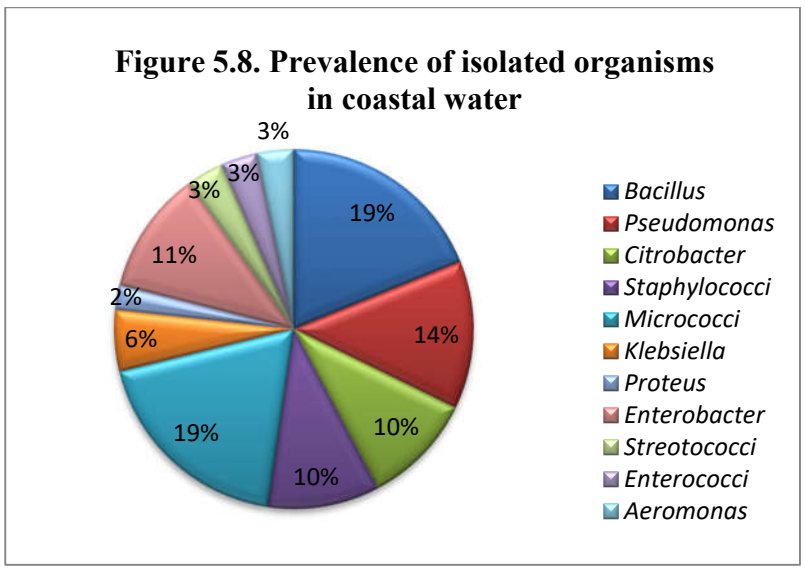
C. 1.2. Selective isolation of target pathogens

Pseudomonas spp and *Aeromonas spp.* were found to be culture positive.

Table 5.25. Selective isolation of target pathogens

Selective media	Pathogens	Interference	Positive samples
Eosin methylene blue (EMB)agar	*E. coli*	Large, blue-black colonies with a green metallic sheen	Nil
King's B agar	*Pseudomonas spp*	Greenish yellow colonies	Positive
Deoxy Cholate agar (DCA)	*Salmonella spp* *Shigella spp*	Colorless colonies with or without black color	Nil Nil
Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar	*V. cholerae*	Yellow colonies	Nil
Starch ampicillin agar (SAA)	*Aeromonas spp*	Produced honey color colonies surrounded by clear zone with Lugol's iodine	Positive
Yersinia selective agar (YSA)	*Yersinia spp*	Dark pink centered colonies	Nil

C. 1.3. Prevalence of Isolated Organisms



The current study isolated and identified a total of 90 isolates from coastal water, among which 18.8 % (n=17) were found to be *Bacillus spp*. and *Micrococci,* 13.3 % (n=12) was found to be *Pseudomonas spp*., 11.1 % (n=10) was *Enterobacter spp*., 10 % (n=9) were *Staphylococcus aureus* and *Citrobacter spp*. In addition to these isolates, a lower percentage of *Klebsiella spp.,* (5.5 %, n=5), *Enterococci, Streptoccci,* and *Aeromonas spp.* (3.3 %, n=3), and *Proteus spp*. (2.2 %, n=2) (Figure5.8). No *E. coli* was isolated during the study.

C. 2. Multiplex PCR

As shown in Figure. 5.9, only four amplicons to the target genes *GyrB, Ahh1, IpaB,* and *UidA* were detected in different combinations. *IpaH* (*Shigella spp*.), *OmpW* (*V. choleare*) or *Ail* (*Y. enterocolitica*) have not been detected by mPCR in the samples. *Gyr B* positive *P. aeruginosa* was detected in 17 samples; *Ahh1* of *A. hydrophila* was identified in 10 samples; followed by *E. coli* with *UidA* were detected in 2 samples and *IpaB* of *S. enterica* were positive for two samples (Figure 5.9 a-c).

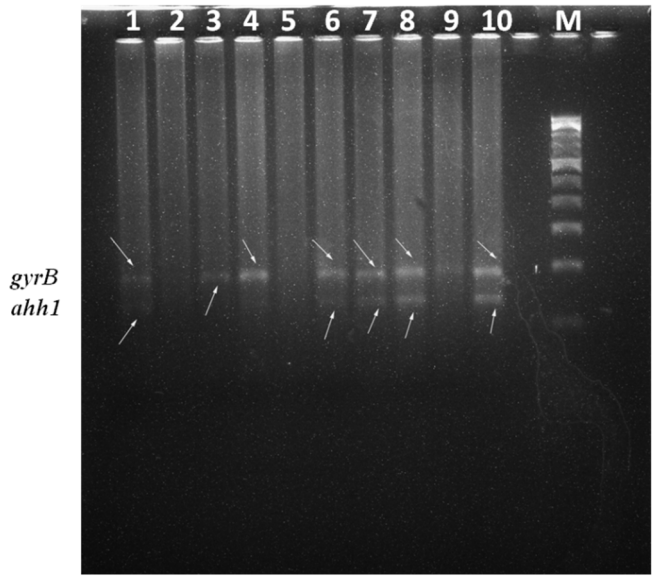


Figure 5.9. (a). Multiplex PCR results of coastal well water samples (1-10 samples) Lane M- 100bp DNA ladder; Lane 1-10-Samples

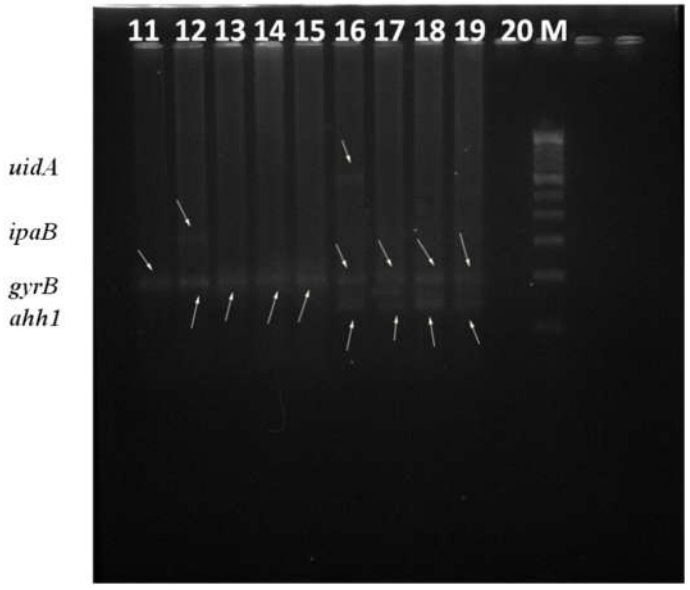


Figure 5.9. (b). Multiplex PCR results of coastal well water samples (11-20 samples) Lane M- 100bp DNA ladder; Lane 11-20-Samples

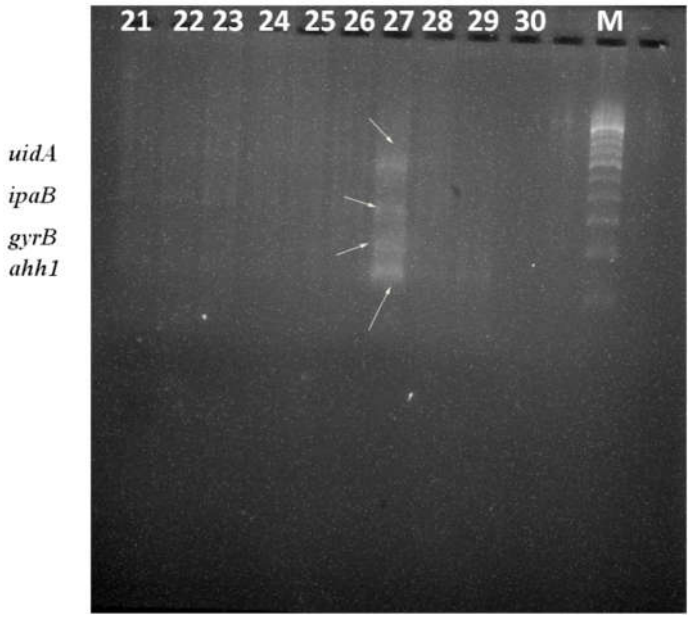


Figure 5.9. (c). Multiplex PCR results of coastal well water samples (21-30 samples) Lane M- 100bp DNA ladder; Lane 21-30-Samples

Quantitative analysis by real time PCR, by using this equation,

$$Q=10^{(Ct-b/m)}$$

Q=Quantity of DNA in the unknown sample

Ct=Ct value of the unknown sample

b=Intercept

m=Slope

C. 3.1. *Pseudomonas aeruginosa*

Quantitative analysis of *Pseudomonas aeruginosa* by real time PCR, by using this equation,

$$Y = -3.122 \text{ (Slope) } \lg X + 34.975 \text{ (Intercept)}$$

Table 5.26. Quantitative analysis of *Pseudomonas aeruginosa*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	22.61/ 23.06	$7.8\times10^3/1.8\times10^3$	16	30.05/28.72	69/44
2	30.22/ 29.70	41/11	17	24.53/24.46	$2.3\times10^3/0.08\times10^3$
3	23.06/ 23.03	$6.6\times10^3/0.10\times10^3$	18	29.19/29.14	73/1.9
4	26.12/24.75	$1.3\times10^3/0.85\times10^3$	19	26.57/26.71	$4.7\times10^2/0.34\times10^2$
5	28.46/29.68	85/51	20	30.09/29.81	41/6
6	27.27/25.04	$9.1\times10^2/8.7\times10^2$	21	29.63/29.91	46/6.7
7	26.80/29.62	$2.3\times10^2/2.6\times10^2$	22	29.34/29.56	59/7
8	21.61/23.00	$1.9\times10^4/0.68\times10^4$	23	30.07/29.56	45/12
9	29.63/29.78	49/3.8	24	29.53/29.28	61/8
10	23.09/25.86	$3.6\times10^3/3.9\times10^3$	25	29.20/30.01	55/23
11	22.31/22.95	$9.3\times10^3/3\times10^3$	26	ND	ND
12	24.95/22.54	$5.6\times10^3/5.6\times10^3$	27	25.23/24.07	$2.2\times10^3/1.3\times10^3$
13	23.42/21.54	$1.3\times10^4/1.1\times10^4$	28	29.76/30.15	40/8.3
14	28.38/30.00	84/63	29	29.86/29.99	41/3
15	28.80/30.04	66/41	30	29.89/29.13	59/23
Positive control		15.06/15.29			$2.2\times10^6/0.3\times10^6$
Non template control-NTC		ND			ND

ND-Not Detected

C.3.2. *Aeromonas hydrophila*

Quantitative analysis of *Aeromonas hydrophila,* by real time PCR, using this equation,

$$Y = -3.223 \text{ (Slope) } \lg X + 37.713 \text{(Intercept)}$$

Table 5.27. Quantitative analysis of *Aeromonas hydrophila*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	17.98/17.90	$1.4\times10^6/0.06\times10^6$	16	23.35/23.52	$2.7\times10^4/0.23\times10^4$
2	33.25/34.70	16.4/11.1	17	23.41/22.89	$3.4\times10^4/0.9\times10^4$
3	31.73/32.81	52.5/27.3	18	16.04/16.08	$5.2\times10^6/0.1\times10^6$
4	36.60/32.14	27.9/36.3	19	14.56/16.40	$1.02\times10^7/0.7\times10^7$
5	30.56/30.49	$1.7\times10^2/0.06\times10^2$	20	39.29/37.56	0.72/0.6
6	15.97/16.51	$4.7\times10^6/1.3\times10^6$	21	31.98/30.52	$1.2\times10^2/0.8\times10^2$
7	24.53/23.25	$2.2\times10^4/1.3\times10^4$	22	33.34/34.83	15.3/10.5
8	22.69/23.55	$3.5\times10^4/1.5\times10^4$	23	28.77/30	$4.21\times10^2/2.5\times10^2$
9	30.87/32.16	92.8/56.5	24	29.28/29.80	$3.5\times10^2/0.9\times10^2$
10	23.11/23.63	$2.8\times10^4/0.7\times10^4$	25	31.62/32.71	56.7/29.7
11	35.12/34.96	6.7/0.5	26	33.19/31.70	49.4/34
12	31.24/30.68	$1.3\times10^2/0.4\times10^2$	27	22.56/23.96	$3.4\times10^4/2.2\times10^4$
13	31.52/31.96	72.2/15.9	28	30.33/30.83	$1.7\times10^2/0.41\times10^2$
14	37.91/35.64	2.6/2.5	29	ND	ND
15	39.14/39.46	0.32/0.05	30	ND	ND

Positive control	16.3/18.39	$2.7\times10^6/2.4\times10^6$
Non template control-NTC	ND	ND

ND-Not Detected

C. 3.3. *E. coli*

Quantitative analysis of *E. coli* by real time PCR, by using this equation,

$$Y = -3.396 \text{ (Slope) } \lg X + 41.086 \text{ (Intercept)}$$

Table 5.28. Quantitative analysis of *E. coli*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	16	25.23/24.74	$5.6\times10^4/1.3\times10^4$
2	ND	ND	17	ND	ND
3	30.09/30.05	$1.8\times10^3/0.033\times10^3$	18	ND	ND
4	ND	ND	19	ND	ND
5	ND	ND	20	ND	ND
6	ND	ND	21	ND	ND
7	ND	ND	22	ND	ND
8	ND	ND	23	ND	ND
9	ND	ND	24	ND	ND
10	ND	ND	25	ND	ND
11	ND	ND	26	ND	ND
12	ND	ND	27	29.63/30.07	$2.1\times10^3/0.4\times10^3$
13	30.22/29.70	$1.9\times10^3/0.5\times10^3$	28	ND	ND
14	ND	ND	29	ND	ND
15	ND	ND	30	ND	ND

Positive control	19.50/19.54	$2.2\times10^6/0.04\times10^6$
Non template control-NTC	ND	ND

ND-Not Detected

C.3.4. *Vibrio cholerae*

Quantitative analysis of *V. cholerae* by real time PCR by using this equation,

$$Y = -3.505 \text{ (Slope) } \lg X + 36.909 \text{ (Intercept)}$$

Table 5.29. Quantitative analysis of *V. cholerae*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	21.09/22.09	$2.5\times10^4/1.1\times10^4$	16	ND	ND
2	23.04/23.35	$8.2\times10^3/1.1\times10^3$	17	ND	ND
3	20.47/22.29	$3.2\times10^4/2.4\times10^4$	18	ND	ND
4	22.97/22.47	$1.1\times10^4/0.2\times10^4$	19	ND	ND
5	ND	ND	20	ND	ND
6	ND	ND	21	ND	ND
7	ND	ND	22	ND	ND
8	ND	ND	23	ND	ND
9	ND	ND	24	ND	ND
10	ND	ND	25	ND	ND
11	ND	ND	26	ND	ND
12	ND	ND	27	ND	ND
13	ND	ND	28	ND	ND
14	ND	ND	29	ND	ND
15	ND	ND	30	ND	ND
Positive control		18.81/19.67			$1.1\times10^5/0.5\times10^5$
Non template control-NTC		ND			ND

ND-Not Detected

C.3.5. *Salmonella enterica*

Quantitative analysis of *Salmonella enterica* by real time PCR, by using this equation,

$$Y = -3.604 \text{ (Slope) } \lg X + 34.718 \text{ (Intercept)}$$

Table 5.30. Quantitative analysis of *Salmonella enterica*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	16	ND	ND
2	ND	ND	17	ND	ND
3	26.98/26.92	$1.43\times10^2/0.03\times10^2$	18	ND	ND
4	ND	ND	19	ND	ND
5	ND	ND	20	26.53/26.87	$1.7\times10^2/0.3\times10^2$
6	ND	ND	21	ND	ND
7	ND	ND	22	ND	ND
8	ND	ND	23	ND	ND
9	ND	ND	24	ND	ND
10	ND	ND	25	ND	ND
11	ND	ND	26	ND	ND
12	24.20/25.05	$6.6\times10^2/2.5\times10^2$	27	23.35/23.25	$1.5\times10^3/0.06\times10^3$
13	ND	ND	28	ND	ND
14	ND	ND	29	ND	ND
15	ND	ND	30	ND	ND

Positive control	23.81/23.98	$1.0\times10^3/0.07\times10^3$
Non template control-NTC	ND	ND

ND-Not Detected

C.3.6. *Yersinia enterocolitica*

Quantitative analysis of *Yersinia enterocolitica* by real time PCR, by using this equation,

$$Y = -3.255 \text{ (Slope) } \lg X + 37.110 \text{ (Intercept)}$$

Table 5.31. Quantitative analysis of *Yersinia enterocolitica*

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	16	ND	ND
2	ND	ND	17	ND	ND
3	ND	ND	18	ND	ND
4	ND	ND	19	ND	ND
5	ND	ND	20	ND	ND
6	ND	ND	21	ND	ND
7	ND	ND	22	ND	ND
8	ND	ND	23	ND	ND
9	ND	ND	24	ND	ND
10	ND	ND	25	ND	ND
11	ND	ND	26	ND	ND
12	ND	ND	27	38.32/38.60	0.8/**0.6**
13	ND	ND	28	ND	ND
14	ND	ND	29	ND	ND
15	ND	ND	30	ND	ND

Positive control	19.51/15.73	$1.9\times10^6/2.4\times10^6$
Non template control-NTC	ND	ND

ND-Not Detected

C.3.7. *Shigella spp.*

Quantitative analysis by real time PCR by using this equation,

$$Y = -3.466 \text{ (Slope) } \lg X + 36.237 \text{ (Intercept)}$$

Table 5.32. Quantitative analysis of *Shigella spp*.

Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)	Sample No.	Ct values (in duplicates)	Quantity of DNA (Copy number)
1	ND	ND	16	ND	ND
2	ND	ND	17	ND	ND
3	ND	ND	18	ND	ND
4	ND	ND	19	ND	ND
5	ND	ND	20	ND	ND
6	ND	ND	21	ND	ND
7	ND	ND	22	ND	ND
8	ND	ND	23	ND	ND
9	ND	ND	24	ND	ND
10	ND	ND	25	ND	ND
11	ND	ND	26	ND	ND
12	ND	ND	27	ND	ND
13	ND	ND	28	ND	ND
14	ND	ND	29	ND	ND
15	ND	ND	30	ND	ND

Positive control	15.50/15.45	$9.8\times10^5/0.2\times10^5$
Non template control-NTC	ND	ND

ND-Not Detected

Table 5.33. Comparison of qPCR, multiplex PCR, and conventional methods- coastal water samples

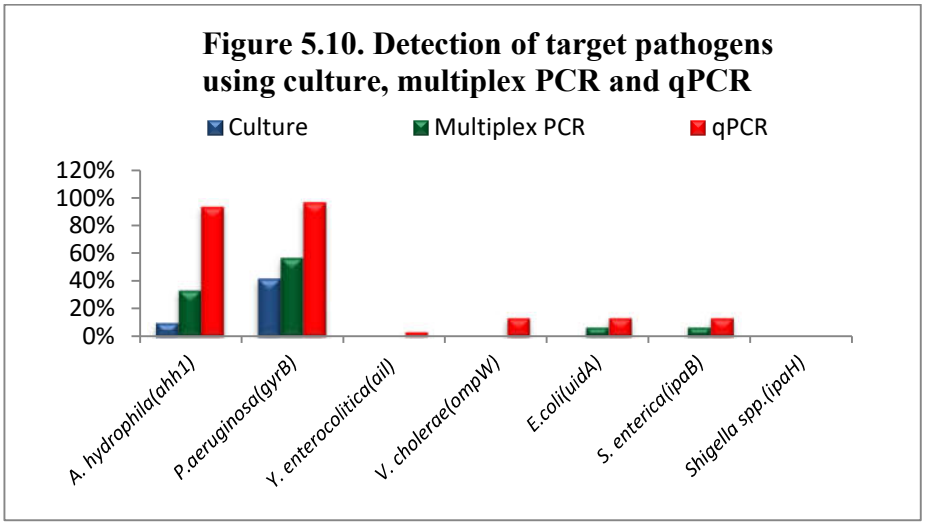
Sl. No	*Pseudomonas spp*			*Aeromonas spp*			*E. coli*			*Salmonella spp*			*Vibrio cholerae*			*Yersinia spp.*			*Shigella spp.*		
	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT	C	M	qRT
1	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
2	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
3	+	+	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-
4	+	+	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
5	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	+	+	+	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
13	+	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
14	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-

17	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
21	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	+	+	+	-	+	+	-	+	+	-	+	+	-	-	-	-	-	+	-	-	-
28	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	**12**	**17**	**29**	**3**	10	28	**0**	**2**	**4**	**0**	**2**	**4**	**0**	**0**	**4**	**0**	**0**	**1**	**0**	**0**	**0**

C= Culture, M=Multiplex PCR, qRT=Quantitative real time PCR.

Table 5.34. Detection of target pathogens using culture, multiplex PCR and qPCR

Techniques	% Detection of target pathogens						
	A. hydrophila (ahh1)	***P. aeruginosa (gyrB)***	***Y. enterocolitica (ail)***	***V. cholerae (ompW)***	***E. coli (uidA)***	***S. enterica (ipaB)***	***Shigella spp (ipaH)***
Culture	10% (3/30)	41.7% (12/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)
Multiplex PCR	33.3% (10/30)	56.6% (17/30)	0% (0/30)	0% (0/30)	6.6% (2/30)	6.6% (2/30)	0% (0/30)
qPCR	93.3% (28/30)	96.6% (29/30)	3.3% (1/30)	13.3% (4/30)	13.3% (4/30)	13.3% (4/30)	0% (0/30)



The *P. aeruginosa* with the *GyrB* gene was found in 41.7 %, 56.6 %, and 96.6 % by culture, multiplex PCR, and qPCR assays followed by *A. hydrophila* carrying the *Ahh1* gene with 10 % by culture methods, 33.3 % bymultiplex and 93.3 % by qPCR assays.

The indicator organism *E. coli* and *S. enterica* were found to be 6.6 % in multiplex PCR and 13.3 % in qPCR, but culture negative. *Y. enterocolitica* has the *Ail* gene and *OmpW* gene carried by *Vibrio*

cholerae, were found to be qPCR positive and for *V. cholerae* it was 13.3 % and for *Y. enterocolitica* it was 3.3 %. *Shigella spp.* having *IpaH* were not detected by any of these methods throughout the sampling period (Table 5.34, Figure 5.10).

5.4. Discussion

The total coliform counts by MPN/100 ml in water samples collected ranged from >1100 MPN/100 ml to 0, and in most samples, a high MPN index was found, which implies significant bacterial contamination following the BIS guidelines; moreover, 63 samples were measured with an acceptable MPN index. Jyothilekshmi *et al.,* (2019) evaluated well water samples from Alappuzha, found that MPN/100 ml was 23-2400. There are no universally acceptable concentrations of organisms, and the most allowable concentrations used by water supply agencies, health departments, and local judiciaries may vary from 100 CFU to 500 CFU/ml. The total coliform counts by MPN/100 ml in water samples collected from well, public water supply, and coastal water were ranged from 1100 to 0, 93 to 0, and 1100 to 0, respectively. The total viable count expressed as Log CFU/ml ranged from these samples was 4.9 to 0, 6.67 to 0, and 6.63 to 0, respectively. These data reveal a high fluctuation in the distribution of heterotrophic populations in the study period. Most of the samples overall viable bacterial counts were within the range of high-risk grade compared to the drinking water standards recommended by WHO (Aryal *et al.*, 2012). The high count may be associated with increased nutrient availability.

The current study isolated and identified a total of 127 isolates from 50 well water samples, among which 18.1 % (n=23) were found to be *Bacillus spp*., 14.17 % (n=18) were *Pseudomonas aeruginosa*, 11.81 % (n=15) were found to be *Citrobacter spp*., 11.02 % (n=14) were *Staphylococcus aureus*, 10.23 % (n=13) were *Micrococc*i. In addition to these isolates, there were lower percentages of *Klebsiella spp*., (7.08 %, n=9), *Proteus spp*., (5.51 %, n=7), *Enterobacter spp*., and *Aeromonas spp*. (4.72 %, n=6), *Providencia spp*., and *Streptococcus spp*. (2.36 %, n=3), *E. coli*, *Enterococci, Morganella spp., Hafnia spp.,* and *Serratia spp.,* (1.57 %, n=2) present in the samples. In the case of public water supply, a total of 85 isolates from 50 public water supply systems, among which 27.05 % (n=23) were found to be *Bacillus spp*., 24.7 % (n=21) was *Micrococci.,* 10.58 % (n=9) were found to be *Klebsiella spp*., 9.4 % (n=8) were *Staphylococcus aureus* and *Proteus spp*. In addition to these isolates, a lower percentage of *Citrobacter spp*., (7.05 %, n=6), *Pseudomonas spp*. (5.88 %, n=5), *Enterobacter spp.* (4.7 %, n=4) and *Enterococci* (1.17 %, n=1). No *E. coli* was isolated during the study. A total of 90 isolates from coastal water, among which 18.8 % (n=17) were found to be *Bacillus spp*. and *Micrococci.,* 13.3 % (n=12) was found to be *Pseudomonas spp*., 11.1 % (n=10) was *Enterobacter spp*., 10 % (n=9) were *Staphylococcus aureus* and *Citrobacter spp*. In addition to these isolates, a lower percentage of *Klebsiella spp.,* (5.5 %, n=5), *Enterococci, Streptoccci,* and *Aeromonas spp.* (3.3 %, n=3), and *Proteus spp*. (2.2 %, n=2). No *E. coli* was isolated from coastal water.

Furthermore, this study also focused on the detection of prevalent water-borne pathogens in these sites. Characterization of isolates was conducted based on Bergy's manual of systematic bacteriology, and the majority of the isolates were gram-negative bacilli may be due to inadequate treatment. Particular attention is paid to our target pathogens, only *E. coli*, *Pseudomonas spp*., and *Aeromonas spp* were culture-positive, and others were culture negative. Therefore, the occurrence of these pathogens raises a concern about the raw drinking water usage. Data are available on the existence of these pathogens globally (Agwaranze *et al.*, 2017). The involvement of such microorganisms in drinking water systems is causing major global problems, most of which lead to severe gastrointestinal diseases (Cabral, 2010; Chitanand *et al.*, 2010; Skariyachan *et al.*, 2015)

Culture methods failed to identify many targets, possibly due to less pathogenic load or nonculturable state of pathogens (VBNC) (Ramamurthy *et al.*, 2014). For all pathogenic microorganisms of concern, there is currently no systematic system of collecting, processing, and analyzing water samples (Bitton, 2014; Zhao *et al.*, 2014).

Well water samples

The *P. aeruginosa* with the *GyrB* gene was the most prevalent in all the well water samples, and it was found to be 30 %, 58 % and 76 % by culturing, multiplex PCR, and real time PCR assays,

respectively. They were followed by *A. hydrophila* carrying the *Ahh1* gene with 12 %, 48 % and 68 % respectively. The indicator organisms *E. coli* was found to be 6 % culture-positive and in multiplex PCR and 12 % in qPCR. *Y. enterocolitica* has the *Ail* gene, *OmpW* gene carried by *V. cholerae*, *S. enterica* with *Ipa B* and *Shigella spp.* having *IpaH* were not detected by culture methods throughout the sampling period and when coming to *S. enterica* no multiplex PCR was found to be positive and the qPCR data showed that 6 %were positive for *IpaB* genes. The results show that no *V. cholerae* were found to be detected by both multiplex PCR and qPCR. Neither *Yersinia spp*. nor *Shigella spp*. were detected in multiplex PCR, but 2 % positive results were shown in qPCR in the case of *Yersinia spp*. However, *Shigella spp*. was not detected in all the samples by all three methods.

Public water supply

The *P. aeruginosa* with the *GyrB* gene was found in 10 %, 16 % and 18 % by culture, multiplex PCR and qPCR assays followed by *A. hydrophila* carrying the *Ahh1* gene with 2 % by both multiplex and qPCR assays and not detected by culture methods. The indicator organisms *E. coli*, *Y. enterocolitica*, *V. cholerae*, *S. enterica* and *Shigella spp.* were not detected by any of these methods.

Coastal water

The *P. aeruginosa* with the *GyrB* gene was found in 41.7 %, 56.6 % and 96.6 % by culture, multiplex PCR and qPCR assays

followed by *A. hydrophila* carrying the *Ahh1* gene with 10 % by culture methods, 33.3 % by multiplex and 93.3 % by qPCR assays. The indicator organisms, *E. coli* and *S. enterica* were found to be 6.6 % in multiplex PCR and 13.3 % in qPCR, but culture negative. *Yersinia enterocolitica* has the *Ail* gene and *OmpW* gene carried by *Vibrio cholerae*, were found to be qPCR positive; for *V. cholerae* it was 13.3 % and for *Y. enterocolitica* it was 3.3 %. *Shigella spp.* having *IpaH* were not detected by any of these methods throughout the sampling period.

Many reports of various multiplex PCR assays were developed for the simultaneous detection of various water-borne bacterial pathogens such as *Salmonella spp., Shigella spp., Aeromonas spp., Vibrio spp*. (Kong *et al.*, 2002; Gilbride *et al.*, 2006). An mPCR assay to differentiate between pathogenic and commensal *E. coli* was reported by Omar & Barnard (2014) from clinical and environmental samples. This study was designed to co-detect the *E. coli* virulence genes, such as *eaeA* (intimin), *stx1* (shiga-like toxin 1), *stx2* (shiga-like toxin 2), *it* (heat labile enterotoxin), *st* (heat stable enterotoxin), *ial* (invasion toxin), *eagg* (enteroaggregative toxin), *astA* (EAST1 toxin), and *bfp* (bundle-forming pili). A multiplex PCR assay developed by Fan *et al.,* (Fan *et al.*, 2008) to detect *Enterohaemorrhagic E. Coli, Shigella spp., Vibrio parahaemolyticus, Salmonella spp.,* and *Pseudomonas aeruginosa*.

A similar approach has been attempted to detect total coliforms and *E. coli* in drinking water by targeting the genes *yaiO,*

uidA and *lacZ* (Molina *et al.*, 2015). A multiplex PCR assay was developed by Bo Li *et al.,* for direct identification of *E. coli, S. flexneri, S. enterica, C. jejuni, Cl. perfringens*, *L. pneumophila*, *L. monocytogenes* and *V. cholerae (*Li *et al.,* 2019*).* R. Kheiri *et al.,* developed two multiplex PCR assays to co detect six water-borne bacterial pathogens such as *E. coli-uidA*, *Shigella –int*, and *P. aeruginosa- gyrB* and *Salmonella –invA*, *V. choleare –ompW* and *coliforms-lac Z* (Kheiri *et al.*, 2016).

Maheux *et al.,* (2014) targeting *LacZ, WecG*, and *16SrRNA* to detect total coliforms and *E. coli* in 100 ml of the potable water sample. Results showed that *LacZ, WecG,* and *16S rRNA* qPCR assays detected 133 (90.5 %), 111 (75.5 %), and 146 (99.3 %) of the 147 total coliforms strains tested, respectively using Real-time multiplex PCR Many studies suggest that real-time PCR is a good practical tool for quantitative analysis of environmental samples and its rapidity and eases compared with traditional methods prefer real-time PCR for environmental sample analysis. In 2017, David I Walker (2017) designed highly specific *E. coli* qPCR using the *ybbW* gene and its comparison with existing methods such as conventional culture methods and qNASBA for environmental water samples. They claimed that this method was first qPCR assay with 100 % target exclusivity.

Several publications have appeared in recent years documenting the rapid real time PCR based detection of water-borne pathogens. Liu *et al*., (2019) developed Taqman real time PCR assays for the detection of *Escherichia coli O157: H7, Listeria*

monocytogenes/ivanovii, Salmonella enterica, Vibrio parahaemolyticus, β-*streptococcus hemolyticus*, *Yersinia enterocolitica, Enterococcus faecalis, Shigella spp., Proteus mirabilis, Vibrio fluvialis, Staphylococcus aureus*, and *Campylobacter jejuni.* Lopes *et al*., in 2018 succeeded in the rapid detection of *E. coli, Salmonella spp.,* and *Staphylococcus aureus*. Our study focused on the existing and emerging water-borne pathogens such as *A. hydrophila, P. aeruginosa, S. enterica, Y. enterocolitica, E. coli, V. cholerae* and *Shigella spp.* in drinking water samples. From the data, it was clear that qPCR assay was the most sensitive, specific and rapid assay than others.

Because of the secretive nature of a viable but nonculturable state of bacteria, it remains a public health threat, and conventional culture methods cannot detect it. Species-specific quantitative real time PCR was widely used to detect these types of pathogens (El-Aziz *et al.*, 2018). Therefore, understanding the viable but nonculturable (VBNC) state of bacteria raised many concerns about accurately identifying and measuring viable bacteria in the water samples. To assess the viability of VBNC bacteria, many researchers have proposed various methods, including real time PCR assays in water microbiology (Liu *et al.*, 2009; Casasola-Rodriguez *et al.*, 2018). Along with major advantages of real-time PCR, such as cost-effectiveness, absence of post PCR analysis, rapidity, time efficiency, fewer template requirement and sensitivity, it also presents many limitations such as costly instrument, the requirement of a standard

curve for absolute quantification etc. Also, multiplex analysis in real time PCR is still limited.

5.5. Conclusion

Based on the results, it can be concluded that the developed qPCR and mPCR assays that can be performed under the same PCR conditions allow quantification and co-detection of multiple water-borne pathogens in a single run when compared to conventional culture methods without any cross-reaction. The qPCR has a high detection level than multiplex PCR and also it showed good agreement with the multiplex PCR. The findings suggest that the techniques apply to routine water monitoring for the rapid detection of possible pathogens than conventional culture methods. Further research will be needed to multiplex in quantitative real time PCR for the rapid detection and quantification of a large number of pathogens.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Waterborne pathogen monitoring is crucial to ensure safe drinking water for human consumption. Conventional culture methods are effective but can yield false-positive results and are time-consuming and laborious as well as unable to retrieve VBNCs from environmental samples. Most conventional methods help determine the presence or absence of pathogens and cell count. To alleviate or prevent waterborne outbreaks and the spread of waterborne pathogens, it is important to rapidly detect waterborne bacteria in environmental water samples. Not only do molecular methods have a high sensitivity and specificity to detect waterborne pathogens, but they can also discern the viability and non-viability of cells because they use different biomarkers at the gene level. Besides, molecular methods for either microbe detection or the quantification of nucleic acid in water overcome some of the problems associated with culture-based approaches, though creating additional challenges simultaneously, such as the detection of free or extraneous DNA to detect as small as one DNA level. The study's main objective was to develop quantitative real-time PCR and multiplex PCR assays for the quantification and co -detection of multiple waterborne pathogens.

The major findings of the study can be summarized as follows:

- Random screening of the 60 drinking water samples in the study area showed 60 % of the samples were positive for coliforms with the standard plate count ranges from 90-8x10^6 CFU/ml.

- The organisms isolated were found tobe *Staphylococcus aureus* (18.1 %), *Bacillus spp* (18.1 %), *Pseudomonas spp* (17.14 %), *Klebsiella spp* (17.14 %), *Enterobacter spp* (10.48 %), *Citrobacter spp* (9.52 %), *E. coli* (8.57 %), and *Shigella spp* (0.95 %) using morphological and biochemical characters.

- The prevalence of *E. coli* in these tested samples showed 8.57 %, indicates recent fecal contamination and may indicate the possible presence of other pathogens.

- Optimum conditions for monoplex PCR and afterward for multiplex PCR were initially standardized. It has been found that the PCR amplicons containing 130, 190, 315, 471, 530, 588 and 611bp in bacterial strains belonging to *Aeromonas hydrophila, Pseudomonas aeruginosa, Salmonella enterica, Yersinia enterocolitica, E. coli, Vibrio cholerae* and *Shigella spp.*

- In monoplex PCR, all the primers were able to amplify the corresponding DNA. No false positive or negative results have been found been found, confirming the exclusivity.

- The detection limits for the seven target pathogens of all the detection methods have been tested. For the assay, cell suspensions containing 10^6-10^0 cells/ml were used for each reference strain. 10^2 cells/ml for *P. aeruginosa*, 10^3 cells/ml for *S. enterica, E. coli* and *V. cholerae*. In the case *Y. enterocolitica, A. hydrophila* and *Shigella spp.* it was found to

be 10^4 cells/ml in case of culture methods, detection limit of multiplex PCR noted was 10^1 cells for *P. aeruginosa* and *Shigella spp*. and 10^2 cells for *A. hydrophila* and *Vibrio cholerae*. In the case of *S. enterica, E. coli* and *Y. enterocolitica,* it was found to be 10^3 cells. The efficiencies of the standard curve of quantitative real time PCR, ranged from 89 to 109 % for the target genes and the analytical sensitivity was at one cell level for all targets.

- When coming to the cost effectiveness of the selected methods, it was clear that qPCR is a more robust, low cost approach than conventional methods. Routine monitoring using these molecular methods helps to reduce assay costs and time requirements.

- Application of the developed methods among well water, public water supply and coastal water, the total coliform counts by MPN/100 ml ranged from 1100 MPN/100 ml to 0, 93 MPN/100 ml to 0 and 1100 MPN/100 ml to 0 respectively. The total viable count expressed as Log CFU/ml ranged from these samples was 4.9 to 0, 6.67 to 0 and 6.63 to 0, respectively.

- In the case of well water samples, the *P. aeruginosa* with the *GyrB* gene was the most prevalent in all the well water samples, and it was found to be 30 %, 58 % and 76 % by culturing, multiplex PCR and real time PCR assays respectively. They were followed by *A. hydrophila* carrying the

Ahh1 gene with 12 %, 48 % and 68 % respectively. The indicator organisms *E. coli* was found to be 6 % culture-positive and in multiplex PCR and 12 % in qPCR. *Y. enterocolitica* has the *Ail* gene, *OmpW* gene carried by *Vibrio cholerae*, *S. enterica* with *Ipa B* and *Shigella spp.* having *IpaH* were not detected by culture methods throughout the sampling period and when coming to *S. enterica* no multiplex PCR was found to be positive and the qPCR data showed that 6 % were positive for *IpaB* genes. The results show that no *V. cholerae* were found to be detected by both multiplex PCR and qPCR. Neither *Yersinia spp*. nor *Shigella spp*. were detected in multiplex PCR, but 2 % positive results were shown in qPCR in the case of *Yersinia spp*. However, *Shigella spp*. was not detected in all the samples by all the three methods.

- In public water supply, the *Pseudomonas aeruginosa* with the *GyrB* gene was found in 10 %, 16 % and 18 % by culture, multiplex PCR and qPCR assays in public water supply, followed by *Aeromonas hydrophila* carrying the *Ahh1* gene with 2 % by both multiplex and qPCR assays and not detected by culture methods. The indicator organisms *E. coli*, *Y. enterocolitica*, *V. cholerae*, *S. enterica* and *Shigella spp.* were not detected by any of these methods.

- The *P. aeruginosa* with the *GyrB* gene was found in 41.7 %, 56.6 % and 96.6 % by culture, multiplex PCR and qPCR assays in coastal water followed by *A. hydrophila* carrying the *Ahh1*

gene with 10 % by culture methods, 33.3 % by multiplex and 93.3 % by qPCR assays. The indicator organisms *E. coli* and *S. enterica* were found to be 6.6 % in multiplex PCR and 13.3 % in qPCR, but culture negative. *Y. enterocolitica* has the *Ail* gene and *OmpW* gene carried by *V. cholerae*, were found to be qPCR positive and for *V. cholerae* it was 13.3 % and for *Y. enterocolitica* it was 3.3 %. *Shigella spp.* having *IpaH* were not detected by any of these methods throughout the sampling period in coastal water samples.

- From above results, we were successful in the development of more sensitive, rapid, and specific qPCR assay for the detection and quantification of waterborne bacterial pathogens and the assay shows detection limit at 1 cell/ml level than multiplex PCR and culture methods.

Conclusions

We successfully developed multiplex and qPCR assays represent a simple, rapid, and powerful tool for the co-detection and enumeration of waterborne bacterial pathogens than conventional culture methods. The developed qPCR and multiplex PCR assays that can be performed under the same PCR conditions allow quantification and co-detection of multiple waterborne pathogens in a single run when compared to conventional culture methods without any cross-reaction.We were successful in the development of more sensitive, rapid and specific qPCR assay for the detection and quantification of

waterborne bacterial pathogens and the assay shows detection limit at 1 cell/ml level than multiplex PCR, the limit of detection was approximately 10^3 cells/ml for *S. enterica, E. coli*, *Y. enterocolitica*, and 10^2 cells/ml for *V. cholerae* and *A. hydrophila*. 10^1 cells/ml detection limits was found in the case of *P. aeruginosa* and *Shigella spp* and culture methodswhere the detection limits was within the range of 10^4-10^3 cells/ml for all the seven targets. Application of these molecular methods especially qPCR in routine water monitoring, and for the effective assessment of water treatment processes which helps to provide more effective risk monitoring of possible threats to public health. The most prevalent organism in the study area were *P. aeruginosa* and *A. hydrophila* with detection levels of 58 %, 76 % and 48 %, 68 % by multiplex PCR and qPCR (well water), 56.6 %, 96.6 % and 33.3 %, 93.3 % by multiplex PCR and qPCR (coastal water) respectively. Further research will be needed to multiplex in quantitative real time PCR for the rapid detection and quantification of a large number of pathogens.

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Publications/Presentations/Proceedings

- **M, Ambili**, and Denoj Sebastian. "**Evaluation of Sensitivity and Cost-Effectiveness of Molecular Methods for the Co-detection of Waterborne Pathogens in India."** ***Marine biotechnology*** *(New York, N.Y.)*, 10.1007/s10126-021-10078-9. **29 Oct. 2021**, **doi:10.1007/s10126-021-10078-9**. **Published by SPRINGER with Impact factor 3.619.**

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ORIGINAL ARTICLE

Evaluation of Sensitivity and Cost-Effectiveness of Molecular Methods for the Co-detection of Waterborne Pathogens in India

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Abstract
Waterborne microbial diseases are regarded as a major public health concern, particularly in nations with poor sanitation, a lack of social awareness, and problems linked with low socioeconomic status. Waterborne pathogen identification using traditional culture methods is time-consuming and labor-intensive. As a result, there is a growing demand for quick pathogen detection technologies. High sensitivity, specificity, and rapidity are all advantages of using molecular techniques like polymerase chain reaction (PCR) in such instances. In this study, we designed multiplex PCR and quantitative real-time PCR (qPCR) assays for the co-detection and enumeration of waterborne pathogens such as *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Yersinia enterocolitica*, *Escherichia coli*, *Vibrio cholerae*, and *Shigella* spp. Specific primers were selected against the virulence and species-specific genes of the seven target pathogens. For all seven target organisms, the detection limits for conventional culture methods were in the range of 10^3–10^4 cells/ml. While employing multiplex PCR method in this study, *Pseudomonas aeruginosa* and *Shigella* spp. have a detection sensitivity of 10^1 cells/ml, *Vibrio cholerae* and *Aeromonas hydrophila* have a detection sensitivity of 10^2 cells/ml, whereas *Salmonella enterica*, *E. coli*, and *Yersinia enterocolitica* have a detection sensitivity of only 10^3 cells/ml. According to our cost–benefit analysis, these molecular technologies are less expensive, with unit analysis costs of ₹52 and ₹173 for qPCR and multiplex PCR, respectively. Furthermore, all of the target genes had a detection limit of 1 cell/ml in qPCR. Because of their speed, sensitivity, specificity, and cost-effectiveness, these multiplex and qPCR assays could be employed for successful co-detection of aquatic pathogens.

Keywords Waterborne pathogens · Co-detection · Simultaneous detection · Enumeration · Drinking water · Multiplex PCR · qPCR

Introduction

Microbial water contamination is a serious public health issue that can result in large-scale outbreaks of waterborne diseases (Szewzyk et al. 2000). Waterborne diseases are frequently reported even in developed countries with strict water quality standards. In 2013, US Centre for Disease Control confirmed outbreaks from drinking water-related (3.17%) and recreational water (6.1%) resources (Centers for Disease Control and Prevention 2013; Hlavsa et al. 2014). In 2017, 71% of the world's population drank water that had been properly managed and was free of contaminants. Even basic drinking water services are in short supply for 785 million people, including 144 million people who rely on surface water. At least 2 billion people around the world consume feces-contaminated water (WHO 2017). Waterborne diseases are more prevalent in low- and middle-income nations located in Africa and Southeast Asia (Johansson et al. 2012).

In India, 37.7 million people are impacted by waterborne infections each year, with 1.5 million children dying from diarrhea, resulting in a $600 million yearly economic impact. Since 2017, over 10,738 people have died as a result of waterborne diseases, with acute bacterial diarrheal infections being the most serious sickness, followed by viral hepatitis and cholera. The state with the highest death rate is Uttar Pradesh, followed by West Bengal, Assam, Odisha, and Madhya Pradesh. Infection risks are increased by open defecation and a lack of suitable sewage disposal technologies (CBHI 2018).

Direct PCR analysis of certain bacteria isolated from water is challenging due to the low concentration of these organisms

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Assessment of bacteriological quality of drinking water from North Kerala, India

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ABSTRACT

The objective of our study was to monitor bacteriological contamination in drinking water from northern districts of Kerala (Malabar) was carried out and also to detect the suitability of water for drinking purpose. Total coilforms can be detected by most probable number method and quantitative analysis through total Viable Count. Sixty drinking water samples were analysed both qualitatively and quantitatively. The total viable count varies from 90 to 8 x 10^6 CFU/ml and three samples have MPN more than 1600/100ml. About 10^5 bacterial isolates obtained from 60 samples comprised of eight species such as *Staphylococcus aureus* (18.1%), *Bacillus Spp.* (18.1%), *Pseudomonas Spp.* (17.14%), *Klebsiella Spp.* (17.14%), *Enterobacter Spp.* (10.48%), *Citrobacter Spp.* (9.52%), *E.coli* (8.57%), and *Shigella Spp.* (0.95%) respectively. And the distribution of *Escherichia coli* in both public water supplies as well as in well water found to be 15.6% and 19.04% respectively. This reveals drinking water in this area is contaminated. So an urgent action is needed to eliminate this issue by conducting planned bacteriological assessment regularly and it helps to provide safe drinking water to public.

KEY WORDS: BACTERIOLOGICAL ASSESSMENT, DRINKING WATER, ESCHERICHIA COLI, MPN, NORTHERN KERALA

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MICROBIOLOGICAL ANALYSIS OF DRINKING WATER FROM MALABAR REGION OF KERALA

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Abstract

Assurance of drinking water safety is a foundation for the prevention and control of waterborne diseases. Developing countries face the problem of safe water provision to its population. A world resources report says: about 70 per cent of India's water supply is seriously polluted with sewage effluents. An UN report has stated that India's water quality is poor and ranked 120th among the 122 nations. Microbial pathogens are one of the major health risks associated with water and waste water. Current study attempted to analyze 45 drinking water samples both qualitatively and quantitatively from different areas of Malabar region of Kerala. Microbiological quality was primarily determined by enumeration of indicator organisms. A laboratory scale qualitative analysis through most probable number (MPN) method was employed. The indicator bacterium Escherichia coli were detected in 9 water samples out of 45 samples as revealed consequently by the presumptive, confirmed and completed tests of MPN method. Most Probable Number (MPN) count ranges between 2-1600MPN /100ml. The number of viable bacteria isolated from all spots varied from 1x10^3 to 8x10^5 CFU/ml. Other Gram negative bacteria found in the samples included, Pseudomonas sp, Klebsiella sp, Enterobacter sp, Citrobacter sp. and Shigella sp. In Kerala the major reason for water-borne and water-related morbidity are poor sanitation and hygiene, inadequate garbage disposal and drainage facilities. People should be educated to consume water free of contamination in the house hold. These might ultimately result in the improvements of the health standard of our population.

Keywords: *Drinking water, Escherichia coli, MPN, Water borne diseases.*

Introduction

The presence of microbial pathogens in polluted, untreated and treated water poses a considerable health risk to the general public[1]. Routine microbiological monitoring of water for pathogenic bacteria is required, as a measure to prevent the spread of water-borne diseases. The spectrum of water-borne infections is also expanding, and many infectious diseases once believed to be conquered are on the rise [2]. Regular monitoring of water-borne pathogens is required to protect public health [3].

The objective of this study is to determine bacterial contamination in drinking water samples collected from Malabar region of Kerala. In Kerala, more than 500 million liters of industrial effluents are being dumped daily into the river besides untreated human wastes [4].

Materials and methods

Collection of water samples from the sites

Forty-five drinking water samples from different sources (well water, bore well water and tap water) were collected aseptically in sterilized containers over a period of eight months January to August 2015 from Malappuram, Calicut, Waynad, Kannur and Kasaragod districts of Kerala. The water samples were processed immediately for microbiological analysis.

Isolation and detection of total count

Total viable count was performed on nutrient agar by means of serial dilution agar plating method [5]. Centrifuged water sample (0.1 ml) was pre-enriched in Luria Bertani broth and were serially diluted. The dilutions of 10^{-6}, 10^{-7},10^{-8} and 10^{-9} were used for plating and the plates were incubated at 37^{O}C for 24hrs.Specific isolation of bacteria using selective media was done. Morphological characteristics of each colony were studied and viable bacterial count (CFU/ml) was enumerated after overnight incubation [6].

Characterization of isolates

Primary identification was carried out by Gram's staining, followed by motility. Secondary identification using biochemical tests- such as Indole, Methyl Red, Vogues Prauskeur, Citrate utilization test, Triple Sugar Iron Agar, Urease, Lysine decarboxylase, catalase, oxidase etc[7].These biochemical tests were performed as per standard microbiological methods according to Bergy's Manual.

Total Coliform Count by Most Probable Number (MPN) Test

In order to assess the domestic pollution level in the study sites the MPN Test was conducted for total coliform count using phenol red lactose broth. The technique involves three successive steps namely, presumptive test, confirmatory test and completed test [8].

Results and Discussion

As summarized in Table 1 below, drinking water resources were contaminated in these regions. The standard plate count which indicates total microbial count in drinking water was in the range of 1x10^3 to