

**DEVELOPMENT OF MOLECULAR METHOD FOR
DETECTION OF VIABLE *ESCHERICHIA COLI*
IN ENVIRONMENTAL WATERS**

HO CHIN FONG



PERPUSTAKAAN
UNIVERSITI MALAYSIA SABAH

UNIVERSITI MALAYSIA SABAH

**A THESIS SUBMITTED IN FULFILLMENT WITH
THE REQUIREMENT FOR THE DEGREE OF
MASTER OF SCIENCE**

Sila dapatkan
Video / CD / Kaset / Slaid / / 314927
di bahagian Media

**SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITI MALAYSIA SABAH
2008**

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS[®]

JUDUL: DEVELOPMENT OF MOLECULAR METHOD FOR DETECTION OF VIABLE *ESCHERICHIA COLI* IN ENVIRONMENTAL WATERS

IJAZAH: SARJANA SAINS (BIOTEKNOLOGI SEKITARAN)

SESI PENGAJIAN: 2004 – 2008

Saya, HO CHIN FONG mengaku membenarkan tesis Sarjana ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:

1. Tesis adalah hakmilik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian saya.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. TIDAK TERHAD



PERPUSTAKAAN
UNIVERSITI MALAYSIA SABAH
Disahkan oleh

 ANITA BINTI ARSAD
PUSTAKAWAN KANAN
UNIVERSITI MALAYSIA SABAH


(Penulis: HO CHIN FONG)

(TANDATANGAN PUSTAKAWAN)

(Penyelia: Dr. Vun Leong Wan)

Tarikh: 6 JUN 2008

Tarikh: 06/06/08

CATATAN: [®] Tesis dimaksudkan sebagai tesis Ijazah Doktor Falsafah dan Sarjana secara penyelidikan atau disertasi bagi pengajian secara kerja kursus dan penyelidikan, atau laporan Projek Sarjana Muda (LPSM)

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries and references, which have been duly acknowledged.

6 JUNE 2008



HO CHIN FONG
-PS04-001-042-

PERPUSTAKAAN
UNIVERSITI MALAYSIA SABAH



UMS
UNIVERSITI MALAYSIA SABAH

CERTIFICATION

TITLE : **DEVELOPMENT OF MOLECULAR METHOD FOR DETECTION OF VIABLE *ESCHERICHIA COLI* IN ENVIRONMENTAL WATERS**

DEGREE : **MASTER OF SCIENCE**

VIVA DATE : **30 APRIL 2008**

DECLARED BY

1. SUPERVISOR

DR. VUN LEONG WAN



2. CO-SUPERVISOR

TEOH PEIK LIN



UMS
UNIVERSITI MALAYSIA SABAH



ACKNOWLEDGEMENT

First and foremost, I would like to thank my supervisors, Dr. Vun Leong Wan of the School of Science and Technology and Ms. Teoh Peik Lin of the Biotechnology Research Institute, Universiti Malaysia Sabah for their guidance and contribution by furnishing me with advices as well as opinions to this research and thesis writing.

I also thank to the Universiti Malaysia Sabah for the generous financial support through a fundamental grant (B-02-04-01-ER/U116) for the research and the Ministry of Science, Technology and Innovation, Malaysia for the offering of scholarship under Pelan Tindakan Pembangunan Teknologi Perindustrian (PTPTP) during my study at Master level.

In addition, I am indebted to Biotechnology Research Institute for the permission to use the machines and apparatuses and conduct the laboratory work of this research in their laboratory. I acknowledge laboratory assistants, Pn. Vidarita and Mr. Richard for their assistance during conducting of laboratory work.

Last but not least, my gratitude goes to my fellow friends who are also conducting their research together in laboratory of Biotechnology Research Institute for their inspiration, idea and advices in laboratory work skills and also Amelia Mavis Lee Ting Ting and Kay Kian Hee for their guidance in thesis writing format.



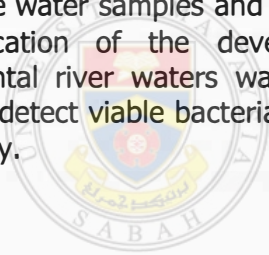
UMS
UNIVERSITI MALAYSIA SABAH

HO CHIN FONG
PS04-001-042
6 JUNE 2008

ABSTRACT

Development of Molecular Method for Detection of Viable *Escherichia coli* in Environmental Waters

Molecular methods are widely used in detection of bacteria in environmental waters. However, most of these methods cannot distinguish between viable and dead cells. Thus, a method for detection of only viable bacteria in environmental waters by DNA-based polymerase chain reaction (PCR) was developed in this study. The developed method consists of DNase treatment to remove dead cells' DNA and 'free' DNA, followed by cell lysis, PCR and agarose gel electrophoresis. A PCR protocol for *Escherichia coli* (*E. coli*) as laboratory model, using primers 16E1/E2, 16E1/E3 or 16E1/E2/E3 was established. In removing dead cells' DNA and 'free' DNA, treatment with DNase I was preferred as compared to washing- centrifugation procedure as it was able to completely remove heat-killed cells' DNA and 'free' DNA from water sample without affecting the DNA in viable cells. Ten units of DNase I was used in this study for complete removal of dead cells' DNA and 'free' DNA and PCR analysis was performed within 1 h after DNase treatment. Results also showed that additional DNase inactivation step was unnecessary after DNase treatment and equivalent concentration of DNase reaction buffer in samples with DNase was included in the controls to obtain accurate comparison. In application of the developed method in environmental waters, membrane filtration (0.45 µm pore size) was used to concentrate water samples and it was found that it could partially remove 'free' DNA. The application of the developed method following membrane filtration in environmental river waters was also feasible. In conclusion, a simple and rapid method to detect viable bacteria in environmental waters was successfully developed in this study.



UNIVERSITI MALAYSIA SABAH

ABSTRAK

*Kaedah molekular digunakan secara meluas dalam pengesanan bakteria di dalam air persekitaran. Namun, kebanyakan kaedah ini tidak boleh membezakan antara sel-sel hidup dan mati. Dengan itu, suatu kaedah bagi pengesanan hanya bakteria hidup di dalam air persekitaran dengan menggunakan tindakan berantai polimerase (PCR) yang berasaskan DNA dibangunkan dalam kajian ini. Kaedah yang dibangunkan ini mengandungi rawatan DNase yang bertujuan menyingkirkan DNA sel-sel mati dan 'DNA bebas', diikuti lisis sel, PCR dan elektroforesis gel agaros. Protokol PCR untuk *Escherichia coli* (*E. coli*) sebagai model makmal, menggunakan primer 16E1/E2, 16E1/E3 atau 16E1/E2/E3 didirikan. Dalam penyingkiran DNA sel-sel mati dan 'DNA bebas', rawatan dengan DNase I menjadi pilihan berbanding dengan prosedur pembasuhan-pengemparan disebabkan DNase I berupaya menyingkirkan DNA sel-sel yang dibunuh oleh haba dan 'DNA bebas' daripada sampel air secara sepenuhnya tanpa memberi kesan kepada DNA di dalam sel-sel hidup. Sepuluh unit DNase I digunakan di dalam kajian ini bagi penyingkiran sepenuhnya DNA sel-sel mati dan 'DNA bebas' dan analisis PCR dijalankan dalam sejam selepas rawatan DNase. Keputusan juga menunjukkan bahawa langkah tambahan penyahaktifan DNase tidak semestinya diperlukan selepas rawatan DNase dan DNase reaction buffer dengan kepekatan yang setara dalam sampel dirawat DNase dimasukkan ke dalam kawalan untuk memperoleh perbandingan yang tepat. Dalam pengaplikasian kaedah yang dibangunkan di dalam air persekitaran, penurasan membran (saiz liang 0.45 μm) digunakan untuk memekatkan sampel air dan ini didapati boleh menyingkirkan sebahagian 'DNA bebas'. Pengaplikasian kaedah yang dibangunkan berikutan penurasan membran di dalam air sungai persekitaran juga adalah boleh dilaksanakan. Kesimpulannya, kaedah yang ringkas dan pantas untuk mengesan bakteria hidup di dalam air persekitaran berjaya dibangunkan di dalam kajian ini.*

UNIVERSITI MALAYSIA SABAH

CONTENTS

	PAGE
TITLE	i
DECLARATION	ii
CERTIFICATION	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
ABSTRAK	vi
CONTENTS	vii
LIST OF FIGURES	xii
LIST OF TABLES	xv
ABBREVIATIONS AND SYMBOLS	xvi
CHAPTER 1 INTRODUCTION	1
CHAPTER 2 LITERATURE REVIEW	5
2.1 MICROBES IN WATER	5
2.2 PHYSIOLOGICAL STATES OF BACTERIA IN WATER	6
2.2.1 Viable and culturable	8
2.2.2 Viable but nonculturable	10
2.2.3 Dead	11
2.3 MICROBIOLOGICAL WATER QUALITY	12
2.3.1 Waterborne diseases	13
2.3.2 Waterborne diseases outbreak incidents	14
2.3.3 Indicator organisms	16
2.4 WATER QUALITY STANDARDS (MICROBIOLOGICAL QUALITY)	17

2.4.1	WHO guidelines	18
2.4.2	US drinking water standards	18
2.4.3	Water quality standards established in Malaysia	19
2.5	DETECTION OF BACTERIA IN WATER	19
2.5.1	Classical methods	20
2.5.2	Enzymatic methods	23
2.5.3	Molecular methods	25
2.6	VIABILITY ASSESSMENT OF DETECTED BACTERIA IN WATER	30
2.6.1	Enrichment-PCR	31
2.6.2	Ethidium bromide monoazide (EMA)-PCR	32
2.6.3	Reverse transcription-PCR (RT-PCR)	34
2.6.4	Nucleic acid sequence-based amplification (NASBA)	35
2.7	IDEAL METHOD FOR DETECTION OF BACTERIA IN WATER	36
CHAPTER 3	METHODOLOGY	38
3.1	PCR OPTIMIZATION FOR <i>ESCHERICHIA COLI</i>	38
3.1.1	Bacterial culture conditions	38
3.1.2	Viable counts	38
3.1.3	Preparation of bacterial lysates for PCR	39
3.1.4	PCR amplification	39
3.1.5	Detection of PCR products	40
3.2	DEVELOPMENT OF METHOD FOR DETECTION OF ONLY VIABLE BACTERIA	40
3.2.1	Preparation of viable cells, heat-killed cells and 'free' DNA	41

3.2.2	DNase treatment	42
3.2.3	Washing procedure in centrifugation	43
3.2.4	Viability verification	44
3.2.5	Preparation of bacterial lysates for PCR	44
3.2.6	PCR amplification	44
3.2.7	Detection of PCR products and DNA	44
3.3	APPLICATION OF THE DEVELOPED METHOD IN ENVIRONMENTAL WATERS	44
3.3.1	Investigation of the effect of filtration on detection of viable cells, heat-killed cells and 'free' DNA	45
3.3.2	Determination of detection limit of the developed method following membrane filtration	47
3.3.3	Analysis of spiked tap and environmental water samples by the developed method	49
3.3.4	Analysis of naturally contaminated environmental water samples by the developed method	51
CHAPTER 4	RESULTS AND DISCUSSION	55
4.1	PCR OPTIMIZATION FOR <i>ESCHERICHIA COLI</i>	55
4.2	DEVELOPMENT OF METHOD FOR DETECTION OF ONLY VIABLE BACTERIA BY PCR	57
4.2.1	Viability verification	58
4.2.2	Effect of DNase on PCR detection of viable cells, heat-killed cells and 'free' DNA	60
4.2.3	Effect of bacteria number or DNA concentration on removing DNA by DNase	65
4.2.4	Inclusion of DNase reaction buffer into controls	70
4.2.5	Omission of DNase inactivation step for samples with DNase	72

4.2.6	Effect of DNase on the DNA stability in prolonged exposure at room temperature	74
4.2.7	Effect of washing steps in centrifugation during sample preparation on PCR detection	79
4.2.8	Overview of method developed to detect viable <i>Escherichia coli</i> in water	82
4.3	APPLICATION OF THE DEVELOPED METHOD IN ENVIRONMENTAL WATERS	82
4.3.1	Effect of filtration on detection of viable cells, heat-killed cells and 'free' DNA	83
4.3.2	Detection limit of the developed method	86
4.3.3	Applicability of the developed method following membrane filtration in artificially contaminated tap and environmental waters	89
4.3.4	Application of the developed method following membrane filtration in detection of only viable cells in naturally contaminated environmental waters	92
4.3.5	General discussion	97
CHAPTER 5	CONCLUSIONS	99
	REFERENCES	103
APPENDIX A	Publication of Partial Results in Proceedings of 5 th KUSTEM Annual Seminar on Sustainability Science and Management, May 2-3, 2006, Terengganu	115
APPENDIX B	Publication of Partial Results in Proceedings of 3 rd Life Sciences Postgraduate Conference, May 24-27, 2006, Universiti Sains Malaysia, Penang	119
APPENDIX C	WHO Guideline Values for Verification of Microbial Quality	124
APPENDIX D	US Primary Drinking Water Standards (Microbiological Quality)	125
APPENDIX E	Microbiological Quality Standards of National Drinking Water Quality Standards (NDWQS)	127

APPENDIX F	Microbiological Quality Standards of Interim National Water Quality Standards (INWQS)	128
APPENDIX G	Preparations of Culture Media, Buffer and Chemicals	129



UMS
UNIVERSITI MALAYSIA SABAH

LIST OF FIGURES

PAGE

Figure 2.1	Decision tree for discriminating different physiological states of individual microbial cells	8
Figure 2.2	The multiple-tube fermentation test	21
Figure 2.3	Repetitive polymerase chain reaction cycles of creating copies of specific fragments of DNA	29
Figure 2.4	Schematic representation of EMA-PCR	33
Figure 3.1	Concentration of bacterial cells in water sample by membrane filtration method	46
Figure 3.2	Steps used in determination of detection limit of the developed method	48
Figure 3.3	Water sampling location: pond at School of Science and Technology, Universiti Malaysia Sabah	50
Figure 3.4	Water sampling location: Likas River	51
Figure 3.5	Water sampling location: Inanam River	52
Figure 3.6	Water sampling location: Petagas River	52
Figure 3.7	Water sampling location: Sembulan River	53
Figure 4.1	PCR amplification of primers 16E1/16E2, 16E1/16E3 and 16E1/16E2/16E3	56
Figure 4.2	DNA extracted from <i>E. coli</i> cells by phenol-chloroform method	60
Figure 4.3	The effect of DNase on viable and heat-killed <i>E. coli</i> cells (3.4×10^8 cfu/ml)	61
Figure 4.4	The effect of DNase on 'free' DNA	62
Figure 4.5	The effect of DNase on viable and heat-killed <i>E. coli</i> cells (9.5×10^8 cfu/ml)	63
Figure 4.6	Hypothetical model that showed the effect of DNase on viable and dead cells' DNA	64
Figure 4.7	Mechanisms of DNA degradation by DNase I	65

LIST OF FIGURES

	PAGE	
Figure 4.8	Effect of bacteria number in removing DNA by DNase in viable cells	66
Figure 4.9	Effect of bacteria number in removing DNA by DNase in heat-killed cells	67
Figure 4.10	Effect of DNA concentration in removing 'free' DNA by DNase	69
Figure 4.11	PCR results of controls without DNase reaction buffer (A) and with DNase reaction buffer (B) to compare with the DNase treated sample respectively	71
Figure 4.12	Effect of omission of DNase inactivation step after DNase treatment on the PCR detection of viable cells	73
Figure 4.13	DNA extracted from <i>E. coli</i> cells by phenol-chloroform method	74
Figure 4.14	PCR detection of DNA in viable cells with DNase (A) and without DNase (B) after leaving at room temperature for 0 min, 30 min, 1 h, 3 h and 24 h	75
Figure 4.15	PCR detection of DNA in heat-killed cells with DNase (A) and without DNase (B) after leaving at room temperature for 0 min, 30 min, 1 h, 3 h and 24 h	76
Figure 4.16	PCR detection of 'free' DNA with DNase (A) and without DNase (B) after leaving at room temperature for 0 min, 30 min, 1 h, 3 h and 24 h	78
Figure 4.17	The effect of washing steps in centrifugation on PCR detection of viable and heat-killed <i>E. coli</i> cells	80
Figure 4.18	The effect of washing steps in centrifugation on PCR detection of 'free' DNA	81
Figure 4.19	The effect of membrane filtration on PCR detection of viable and heat-killed <i>E. coli</i> cells	84
Figure 4.20	The effect of membrane filtration on PCR detection of 'free' DNA of <i>E. coli</i> cells	85
Figure 4.21	Analysis of the detection limit of the developed method by using primer 16E1/16E2/16E3	87

LIST OF FIGURES

	PAGE	
Figure 4.22	Analysis of the detection limit of the developed method by using primer LZL-389/LZR-653	87
Figure 4.23	Application of the developed method following membrane filtration in artificially contaminated laboratory tap water (A), SST pond water (B) and Likas river water (C)	91
Figure 4.24	PCR products with target gene generated by using primer pairs LZL-389/ LZR-653 and URL-301/ URR-432 in Likas River water (A), Petagas River water (B), Sembulan River water (C) and Inanam River water (D)	94
Figure 4.25	Growth of coliform (dark purple to black with dark centers and pink colonies) and <i>E. coli</i> (green metallic sheen colonies) in Likas River water(A), Petagas River water (B), Sembulan River water (C) and Inanam River water (D) which were diluted in 10^{-2} before plating on EMB agar	95



UMS
UNIVERSITI MALAYSIA SABAH

LIST OF TABLES

		PAGE
Table 2.1	Major waterborne diseases outbreak in Malaysia	15
Table 2.2	Examples of worldwide major waterborne disease outbreak cases	15
Table 2.3	Advantages and disadvantages of commonly used fecal indicators	17
Table 3.1	Preparation of PCR mixture for primer pair 16E1/E2, 16E1/E3 and 16E1/E2/E3 respectively	39
Table 3.2	Preparation of PCR mixture for primer pair LZL-389/LZR-653	49
Table 3.3	Preparation of PCR mixture for primer pair URL-301/URR-432	54
Table 4.1	Visible turbidity of bacterial growth in viable and heat-killed water samples in all the experiments associated with DNase	59
Table 4.2	Visible turbidity of bacterial growth in viable and heat-killed water samples with the inclusion of two washing steps in centrifugation	59
Table 4.3	Visible turbidity of bacterial growth in viable and heat-killed samples at respective time points	60

ABBREVIATIONS AND SYMBOLS

ABNC	Active but nonculturable
AMV-RT	Avian myeloblastosis virus reverse transcriptase
APHA	American Public Health Association
bp	base pair
Ca ²⁺	Calcium ion
cDNA	complementary deoxyribonucleic acid
cfu	colony forming unit
CFUs	Colony-forming units
CTC	5-cyano-2,3,-ditolyl tetrazolium chloride
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DOE	Department of Environment
DVC	Direct viable count
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
EMA-PCR	Ethidium bromide monoazide-Polymerase chain reaction
EMB	Eosin Methylene Blue
EPA	Environmental Protection Agencies
FISH	Fluorescent in situ hybridization
GWUDI	Ground Water Under Direct Influence
h	hour
HPC	Heterotrophic plate count
IFA	Immunofluorescence assay
IMS	Immunomagnetic separation
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-2H tetrazolium chloride
INWQS	Interim National Water Quality Standards
ISH	In situ hybridization

LB	Luria-Bertani
MCL	Maximum contaminant level
MF	Membrane filter
Mg ²⁺	Magnesium ion
MgCl ₂	Magnesium chloride
mg/L	milligrams per liter
mg/ml	milligram per milliliter
min	minute
ml	milliliter
μl	microliter
μm	micrometer
μM	micromolar
mM	millimolar
mQ	milli Q
mRNA	messenger ribonucleic Acid
MTF	Multiple-tube fermentation
NASBA	Nucleic acid sequence-based amplification
NDWQS	National Drinking Water Quality Standard
NTU	Nephelometric turbidity units
PCR	Polymerase chain reaction
RNA	Ribonucleic Acid
RNase H	Ribonuclease H
rRNA	Ribosomal ribonucleic Acid
RT-PCR	Reverse transcription-Polymerase chain reaction
s	second
SDS	Sodium dodecyl sulfate
TAE	Tris/acetate/EDTA
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
Tris-HCl	Trishydroxymethylaminomethane Hydrochloride
U	Unit
US	United States
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
V	Volt

VBNC	Viable but nonculturable
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organization
x g	x gravity
Å	Angstrom
β	Beta
°C	Degree Celsius
>	More than
<	Less than
≈	Approximately



UMS
UNIVERSITI MALAYSIA SABAH

CHAPTER 1

INTRODUCTION

Water is one of the earth's most precious and threatened resources. It is essential to sustain life for plants, animals and humans. It favors the existence of many types of microbes such as bacteria, archaea, protozoa, micro-algae, fungi and virus (Prescott *et al.*, 1999). Some of these microbes are pathogenic and can affect human health through waterborne diseases. Examples of common waterborne diseases caused by bacterial pathogen are cholera, typhoid, salmonellosis, infectious hepatitis and campylobacteriosis. Outbreaks of waterborne diseases had resulted in large number of fatalities worldwide. According to rough estimates, annually more than 15 million deaths worldwide resulted from waterborne infections in the past (Atlas and Bartha, 1998).

Since water is vital to our survival and its microbiological quality has great bearing on health, it is important to ensure the safety and microbiological quality of water. Various environmental legislation and standards relating to microbiological water quality which are usually mandatory with maximum permissible concentrations based on health criteria or environmental quality standards have been established in different countries for this purpose (Gray, 1999). To comply with these regulatory requirements for protection of public and environmental health, there are necessitates monitoring water for various types of microbial pathogens or indicator organisms.

To date, there are a number of established methods and emerging approaches for the detection of bacterial pathogens or indicator bacteria in water (Rompre *et al.*, 2002). These methods include classical methods, enzymatic methods and molecular methods. The conventional or classical methods routine used for detecting bacteria are mainly culture-based methods (Rompre *et al.*, 2002). These

methods rely on a pre-enrichment of bacteria in a non-selective media and/or culturing the bacteria in selective media and identifying isolates according to their morphological or biochemical/immunological characteristics by biochemical or serological test on grown colonies (Sheridan *et al.*, 1998). These methods are sensitive and can allow small numbers of bacteria to be detected. However, there are several problems with culturing methods used for routine monitoring of the bacteriological safety of water. These include time consuming, results are obtained only several days after receiving the water sample, difficulty of interpretation of identification schemes based on phenotypic properties and might result in specificity problems and failure to isolate viable but nonculturable bacteria which may also pose health hazards (Atlas and Bartha, 1998; Sheridan *et al.*, 1998; Min and Baeumner, 2002).

Enzymatic method is an attractive alternative to classical methods. In enzymatic methods, defined substrate technology is used to determine enzymatic activities that are diagnostic of bacteria. Detecting and enumerating bacteria through specific enzymatic activities has been widely accepted as the enzymatic reactions are sensitive and rapid. However, it is more expensive and it does not detect viable but nonculturable bacteria. Given the drawbacks of culturing methods and enzymatic methods, alternative methods which do not require cultivation of the target bacteria have been developed. One of the most promising methods is molecular methods.

The use of molecular methods such as immunological methods and nucleic acid-based methods is growing rapidly in the environmental microbiology field. Unlike the conventional culture-based methods, these methods especially nucleic acid-based methods are based on the detection of a fraction of the genetic material of the targeted bacteria (Prescott *et al.*, 1999). The primary advantage of the application of these methods for the detection of bacteria in water is the ability to specifically, sensitively and rapidly detect the bacteria of concern without having to actually isolate it on growth media (Koster, 2002; Keer and Birch, 2003). However, most of these methods will not be able to assess the viability of the detected bacteria since the detected bacteria can be in different physiological states such as viable, viable but nonculturable (VBNC) or dead (Kell *et al.*, 1998).

Due to the importance of viability assessment for interpreting the result of detecting pathogenic bacteria in terms of public health, an ideal method which is not only specific, sensitive, rapid, simple and cost effective but also able to assess viability is necessary to effectively detect bacteria in water and ensure the safety of water system. Many nucleic acid-based approaches based on DNA or mRNA as viability indicator have been developed and investigated for this purpose. This includes enrichment/PCR (Delabre *et al.*, 1998; Tsen *et al.*, 1998; Preez *et al.*, 2003), ethidium bromide monoazide (EMA)-PCR (Nogva *et al.*, 2003; Rudi *et al.*, 2005), reverse transcription-PCR (RT-PCR) (Bej *et al.*, 1996; Herman, 1997) and nucleic acid sequence-based amplification (NASBA) (Birch *et al.*, 2001; Min and Baeumner, 2002) and so on. However, there are still disadvantages or limitations in these methods such as inability to detect viable but nonculturable bacteria by enrichment/PCR, health risk caused by EMA-PCR, difficulty in extraction of mRNA due to the instability of them while using mRNA targeted RT-PCR and NASBA.

In this study, a method which allows the selectively detection of only viable bacteria in water by polymerase chain reaction (PCR) was developed. DNA-targeted PCR method was used because it is specific, sensitive, simple, provides results in a short period of time and has been recognized to play an increasingly important role in the detection of pathogenic bacteria for health care and environment monitoring among all methods (Delabre *et al.*, 1998; Birch *et al.*, 2001). Although DNA persists in a PCR-detectable form after cell death, it was preferred as the target nucleic acid in relation to living versus dead cells study rather than mRNA. This is because it is difficult to detect mRNA in viable bacterial cells due to the instability of mRNA molecule and also there are problems associated with detection of mRNA in injured, stressed and VBNC cells as these cells may contain mRNA in quantities too low for detection (Birch *et al.*, 2001). To develop a PCR method which selectively detects viable but not dead bacterial cells, the approach of removal of dead cells' DNA from sample prior to PCR was applied in this study.

In overall, the objective of this study was to develop a feasible method for the selective detection of viable bacterial cells but not dead cells in environmental waters. In this study, *Escherichia coli* (*E. coli*) was used as a laboratory model, since *E. coli* is a common organism in environmental water and indicator of fecal contamination.

The specific objectives of this study were summarized as follows:-

1. To develop a PCR method which allows detection of only viable *E. coli* in water.
2. To apply the developed method in detection of *E. coli* in environmental waters.



UMS
UNIVERSITI MALAYSIA SABAH

CHAPTER 2

LITERATURE REVIEW

2.1 MICROBES IN WATER

Water covers seven tenths of the Earth's surface and occupies an estimated volume of $1.38 \times 10^9 \text{ km}^3$ (Sigeo, 2005). Water is essential to sustain life for plants, animals and humans. It provides a unique physical environment which is called aquatic environment as habitat for various organisms.

Water can be classified into two categories; surface waters and groundwaters. According to Gray (1999), surface water is a general term describing any water body which is found flowing or standing on the surface, such as streams, rivers, ponds, lakes and reservoirs. Meanwhile, groundwater is water in gravel beds and fractured rocks below the surface soil (Prescott *et al.*, 1999). Surface water and groundwater is important as water sources for drinking water which is normally referred to the water suitable for drinking. These waters in their natural state are treated to remove contaminants prior to be used for drinking. Water is also categorized into fresh water and saline water based on physico-chemical conditions (Gray, 1999). However, we generally conclude these waters which exist in our environment as environmental waters. River water, stream water, lake water, pond water and reservoir water are some common environmental waters (Prescott *et al.*, 1999).

As water provides a unique physical environment, it favors the existence of many types of microbes. Microbes or microorganisms may be defined as those organisms that are not readily visible to the naked eye, requiring a microscope for detailed observation (Sigeo, 2005). These include bacteria, archaea, protozoa, microalgae, fungi and virus. Microbes especially bacteria found in water may be due to numerous sources, including humans, animals, agricultural runoff, industrial waste water, improper waste disposal and failed septic systems (Scott and Rose, 2002).