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

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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 treament 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.

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 polymerase (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 dirawati 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. Kesimpulanya, kaedah yang ringkas dan pantas untuk mengesan bakteria hidup di dalam air persekitaran berjaya dibangunkan di dalam kajian ini.

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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
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorragic *Escherichia coli*
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	Nephelolometric 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

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, archea, 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.

CHAPTER 2

LITERATURE REVIEW

2.1 MICROBES IN WATER

Water covers seven tenths of the Earth's surface and occupies an estimated volume of 1.38×10^9 km^3 (Sigee, 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 (Sigee, 2005). These include bacteria, archea, protozoa, micro-algae, 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).