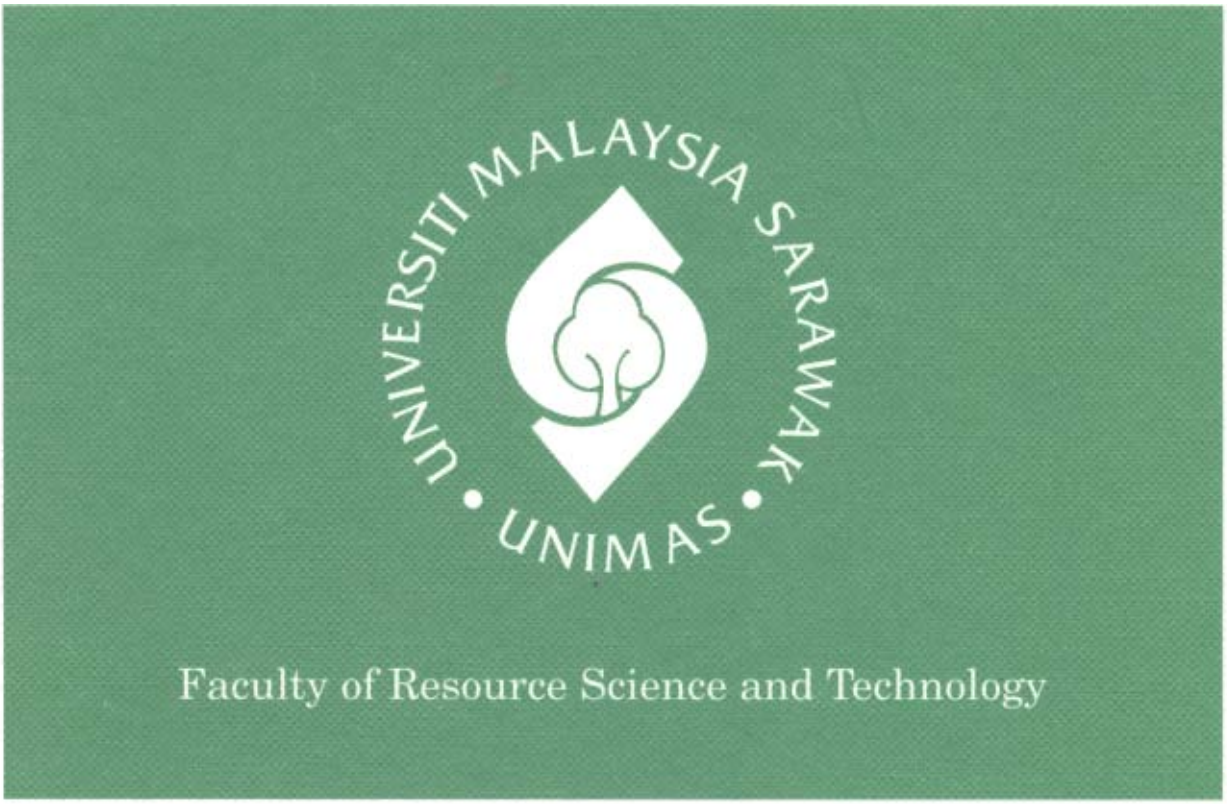

COMPARISON OF DIFFERENT BACTERIAL GENOMIC DNA EXTRACTION PROTOCOLS FOR SELECTED GRAM NEGATIVE BACTERIA

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Bachelor of Science with Honours
(Resource Biotechnology)
2006

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Comparison Of Different Bacterial Genomic DNA Extraction Protocols For Selected Gram Negative Bacteria

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ABSTRACT

In this study, four different DNA extraction protocols for Gram negative bacteria were conducted and compared in terms of its simplicity, quality, cost and rapidness. The bacterial genomic DNA extraction protocols that were evaluated included the phenol/chloroform/isoamyl alcohol (PCI), cetyltrimethylammonium bromide (CTAB), the use of the Wizard® Genomic DNA Purification kit (Promega, USA) and finally with an in-house technique called the boiling cell method. The selected Gram negative bacteria used in this study were *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Escherichia coli*. Serial dilutions were performed on the selected bacteria and subsequently use in colony counting on total plate count agar to determine the number of bacterial cells. Suitability of the yields varies among the protocols but DNA extraction using the Promega kit contained least contaminant in the final extraction products. The genomic DNA obtained was determined via further downstream processes such as specific PCR and enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR). The comparison of these DNA extraction protocols has provided the best method to be performed before engaging other molecular works.

Keywords: DNA extraction protocol; Gram negative bacteria; serial dilution; specific PCR; ERIC-PCR.

ABSTRAK

Dari penyelidikan ini, empat kaedah pengekstrakan DNA bagi bakteria Gram negatif dijalankan dan dibandingkan dari segi keringkasan, qualiti, kos dan masa. Kaedah pengekstrakan DNA bakteria yang dilakukan ialah phenol/chloroform/isoamyl alcohol (PCI), cetyltrimethylammonium bromide (CTAB), Wizard® Genomic DNA Purification kit (Promega, USA) dan kaedah pendidihan. Gram negatif bakteria yang dipilih dalam penyelidikan ini merupkan Vibrio cholerae, Vibrio parahaemolyticus dan Escherichia coli. Pencairan bersiri dijalankan pada Gram negatif bakteria dan seterusnya digunakan untuk pengiraan koloni pada total plate count agar bagi mengenalpasti bilangan koloni dalam kultur bakteria. Secara konsisten, dalam penyelidikan ini mendapati DNA yang diperoleh berubah-ubah berdasarkan kaedah pengekstrakan DNA yang berbeza. Namun secara keseluruhannya, kaedah pengekstrakan dengan kit Promega memberi kesan benda asing yang kurang pada DNA yang diperoleh. Genomik DNA yang diperoleh digunakan untuk analisis seperti specific PCR dan enterobacterial repetitive intergenic consensus (ERIC-PCR). Perbandingan antara kaedah-kaedah pengekstrakan DNA ini memberi pemilihan kaedah terbaik sebelum memulakan penyelidikan molekular.

Kata kunci: *Kaedah pengeksrakan DNA; bakteria Gram negatif; pencairan bersiri; specific PCR; ERIC-PCR.*

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LIST OF ABBREVIATIONS

bp	base pair
cm	centimeter
cfu	colony forming unit
ddH_2O	double distilled water
dNTPs	deoxynucleotide triphosphate
DNA	Deoxyribonucleic Acid
EDTA	ethylenediamine tetra-acetic acid
g	gram
kbp	kilobase pair
K-Ac	potassium acetate
LB	Luria Bertani
m	meter
min	minute(s)
mol	mole
M	Molar or Molarity
mM	miliMolar
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
pmol/ml	picomol per milliliter
PCI	phenol-chloroform-isoamyl alcohol
rpm	revolution per minute
sdH_2O	sterile distilled water
SDS	Sodium Dedocyl Sulphate
Taq	*Thermus aquaticus* DNA Polymerase
TBE	Tris-Borate EDTA electrophoresis buffer
TE	Tris-EDTA buffer
Tris	Tris (hydroxymethyl) methylamine
UV	Ultraviolet
V	volts
wt/vol	weight per volume
µl	microliter
%	Percentage
^oC	degree Celcius
>	more than
<	less than

CHAPTER 1

INTRODUCTION

1.1 Introduction

Genomic studies have shown rapid development over the past years and it is broadly applied in various fields nowadays especially in the field of molecular biology. The widespread use of molecular biological methods has resulted in a dramatic increase in the knowledge of composition and physiology of organisms. Molecular biological methods offer advantages that include the studies of microbial diversity as well as detection of wider range of organisms. There are various tools use and one of the most important is the DNA extraction protocols.

DNA extraction is one of the established methods in molecular biology as only upon DNA extraction can the DNA be liberated to be studied. The purified DNA can then be applied to study DNA structure and chemicals, examine DNA-protein interactions, DNA hybridizations, amplification using polymerase chain reaction (PCR) and gene cloning (McOrist *et al.*, 2002). There are many different DNA extraction methods that have been practised including cycles of freezing and thawing, sonication, boiling, liquid nitrogen, bead beating, SDS, lysozymes, phenol-chloroform (Lipathay *et al.*, 2004) and DNA extraction commercial kits. DNA extractions are routinely applied in many areas of bacterial physiology, genetics, molecular biology and biochemistry. The rapid isolation and analysis of DNA have become fundamental to a variety of diagnostics, forensic and research (Kephart, 1998). According to Johnson (1991), first few individuals who brought nucleic acid isolation protocols were Kirby in 1957 and Marmur in 1961.

The amount of DNA extracted is dependent on the methods used, microbial community composition and characteristic of organisms (Guthrie *et al.*, 2000). Nevertheless, there are also possibilities that variation of results may exist between different methods with the use of different bacterial species (McOrist *et al.*, 2002). In order to obtain good yield from DNA extraction methods, several factors have been studied and need to be improved including the period of time involved, loss of DNA during the extraction procedures, contamination of polysaccharides and other cellular components, difficulties in cell disruption and the degradation of DNA or RNA (Johnson, 1991).

In this study, DNA extraction protocols for three Gram negative bacteria species were compared. The selected representatives are *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Escherichia coli*. Gram negative bacteria are easily lysed by detergents such as sodium dedocyl sulphate (SDS) or by sodium hydroxide (Grimont and Grimont, 1991) as compared to gram positive bacteria. The four DNA extraction methods performed on the Gram negative bacteria were the phenol/chloroform/isoamyl alcohol (PCI) protocol (Ausubel *et al.*, 1990), the CTAB protocol (Ausubel *et al.*, 1990), the cell boiling protocol and the use of Promega Wizard® Genomic DNA Purification kit (Promega, USA). These comparisons take into account the duration of time, financial and technical factors involved in executing each of the selected methods. These protocols were examined and compared based on the effectiveness for extracting genomic bacterial DNA from Gram negative bacteria by using a spectrophotometer, specific PCR (Polymerase Chain Reaction) amplification and ERIC-PCR (enterobacterial repetitive intergenic consensus sequence) analysis of the extracted DNA. The primers targeting outer membrane protein (*ompW*) gene in *V. cholera* (Nandi *et al.*, 2000), thermolabile hemolysin (*tl*) gene in *V. parahaemolyticus* and *uidA* gene in *E. coli* were used in this study.

Selecting the best DNA extraction method for Gram negative bacteria for future studies could mean savings in time, cost and man-power. By doing this study, it may benefit future researches as references and recommendations on the choice of DNA extraction methods for different PCR applications such as specific PCR and ERIC-PCR exclusively for Gram negative bacteria.

1.2 Objectives

The objectives of this study were:

1. To compare the four DNA extraction protocols based on their rapidness and sensitivity.
2. To compare which protocol can give the best DNA purity and quantity with the use of spectrophotometer.
3. To determine the best DNA extraction method for Gram negative bacteria which can give the best and most suitable genomic DNA product for different downstream molecular biology applications such as specific PCR and ERIC-PCR.

CHAPTER 2

LITERATURE REVIEW

2.1 Gram negative bacteria

Gram negative bacteria are unicellular prokaryotic microorganisms. Examples of these type of bacteria are *Escherichia coli* and *Vibrio species*. Gram negative bacteria contain thin layer of peptidoglycan and their cell wall are made of 15-20% peptidoglycan which is intermittently cross-linked. Therefore, with these characteristics, extraction of DNA on these bacteria can be easily achieved.

2.1.1 *Vibrio cholerae* and the outer membrane protein (*ompW*) gene

Vibrio cholerae is from the family *Vibrionaceae*. It is a facultative anaerobe bacterium and can be found in abundance from aquatic or estuarine sources. It is well known for causing cholerae upon consumption of contaminated food and water containing pathogenic *V. cholerae*. *V. cholerae* is widely studied, manipulated and developed to produce vaccines (Drasar, 1997).

There are up to 10 major outer membrane proteins in *V. cholerae*. One of them is called *ompW* gene. The *ompW* gene of *V. cholerae* is produced in small amount and its function in *V. cholerae* is unknown. This *ompW* gene can be used to detect the presence of *V. cholerae*. The *ompW* gene is also found in *E. coli* and has significant sequence similarities to the *ompW* gene of *V. cholerae* as well (Holger *et al.*, 1999).

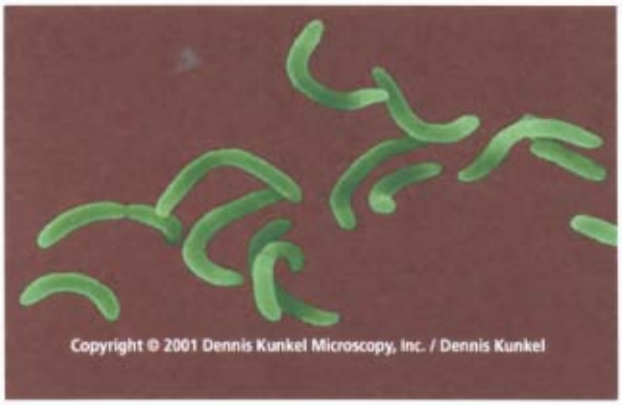

Figure 1. Electron micrograph of *Vibrio cholerae*. (Kunkel, 2001)

2.1.2 *Vibrio parahaemolyticus* and the thermolabile hemolysin (*tl*) gene

Vibrio parahaemolyticus is a halophilic Gram negative bacterium that can cause gastroenteritis in humans. *V. parahaemolyticus* can normally be isolated from warm coastal and estuarine waters (Drasar, 1997). Normally *V. parahaemolyticus* is associated with food poisoning by contaminated seafood consumption and it was first identified in Japan as a cause of food poisoning associated with shrimps (Nandi *et al.*, 2000), raw or partially cooked fishes, shellfish (Marshall *et al.*, 1999) and oysters (Khan *et al.*, 2002). These food poisoning are most commonly occurring in Japan and Southeast Asia. Based on a study done by Nandi *et al.* (2000) the thermolabile (*tl*) hemolysin gene can be used to detect and confirm the presence of *V. parahaemolyticus*.

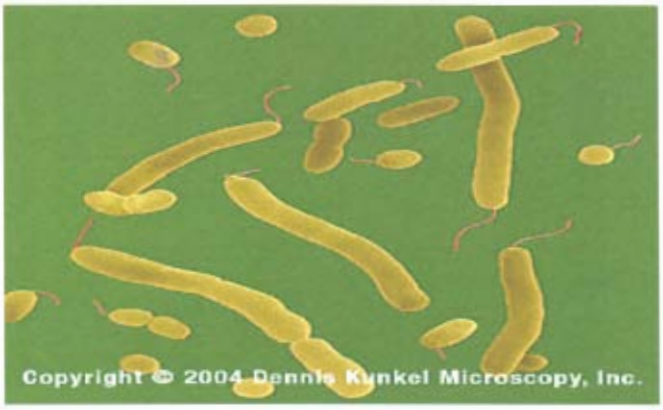

Figure 2. Electron micrograph of *Vibrio parahaemolyticus*. (Kunkel, 2001)

2.1.3 *Escherichia coli* and the β-glucuronidase (*uidA*) gene

Escherichia coli is the most broadly studied of all bacterial species and it was first described by Dr. Theodor Escherich in 1885 (Thielman and Guerrant, 1997). It is from the family of *Enterobacteriaceae*. *E. coli* is mostly found in the large bowel and tissues of warm-blooded animals. It is also found in faecal contaminated soil and water, making it water faecal pollution indicator for many years (Thielman and Guerrant, 1997). *E. coli* are usually harmless commensal and only some strains adhere to the intestinal mucosa whereas others are transient in the gut lumen. McDaniels *et al.* (1996) described that the *uidA* gene can be used for detecting *E. coli*. In the study of Jefferson *et al.* (1980), they have determined the complete nucleotide sequence of *E. coli uidA* gene, encoding β-glucuronidase (GUD). The *uidA* gene genotypic assays are sensitive to detect the presence of *E. coli* isolated from water samples and has frequently been used as probes that codes the enzyme β-glucuronidase. According to Martins *et al.* (1993), most of the *E. coli* carries sequences of *uidA* gene regardless of the β-glucuronidase phenotype.

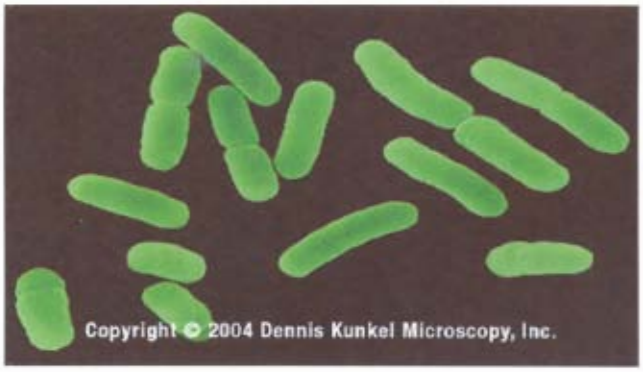

Figure 3. Electron micrograph of *Escherichia coli*. (Kunkel, 2001)

2.2 DNA extraction

The successful application of molecular techniques relies on the effectiveness recovery of DNA from samples. Therefore a reproducible, simple and rapid protocol for DNA extraction is essential for molecular studies. Based on a previous study done by Guthrie *et al.* (2000), the chosen DNA extraction method must result in disruption of all cells, while at the same time, the isolated and purified DNA for amplification are not degraded. Therefore, physical and chemical forms of lysis are recommended to minimize any bias associated with the extraction method. The practical steps in performing DNA extraction are divided into four stages (Brown, 1990). Firstly the bacteria is cultured for growth in a rich medium and harvested by centrifugation (Burden and Whitney, 1995). Secondly, the cells are lysed to release its content and this can be done by exposure to chemical agents that can disrupt the cell membrane. Thirdly, the extracted cells are then treated with Proteinase K and phenol-chloroform to remove all components except the DNA. Finally, the resulting DNA solution is concentrated by ethanol precipitation. Once genomic DNA is extracted from the bacteria, it can be manipulated using standard techniques (Brown, 1990).

2.2.1 Phenol/Chloroform/Isoamyl alcohol protocol

The most general DNA extraction method commonly used is the phenol/chloroform/isoamyl alcohol (PCI) extraction protocols modified from the procedures of Marmur (1961) and Kirby (1967) (Johnson, 1991). According to McOrist *et al.* (2002), DNA extraction using PCI method takes time as additional clean-up procedure are required to remove contaminants which can inhibit the PCR reaction. Brown (1990) mentioned that Proteinase K breaks polypeptides down into smaller units that can be easily remove by phenol. The use of chloroform and phenol improves the efficiency of the extraction process to denature proteins and removes lipid from the sample. Moreover, its high density characteristic makes separation of phases easier in the extraction process, making the removal of protein easier (Burden and Whitney, 1995). The isoamyl alcohol added to the chloroform prevents it from foaming (Webb and Wilson, 1991) and also helps in the separation of organic phases. Since the phenol residue in the aqueous phase can interfere with downstream manipulations of DNA, the DNA is often precipitated by addition of isopropanol prior to washing with ethanol.

2.2.2 Cetyltrimethylammonium bromide (CTAB) protocol

The cetyltrimethylammonium bromide (CTAB) protocol has evolved from the works of Jones in 1953. Johnson (1991) described that cetyltrimethylammonium ion is a cationic detergent, therefore recommending it not to be used with SDS or phenol because mixtures of totally insoluble complexes will form. In the presence of CTAB, DNA is soluble if there is also a high concentration of monocovalent cations such as Na^+ or NH_4^+. From this step, proteins can be dissociated from the nucleic acids. This protocol is used for recalcitrant organisms that need to be physically disrupted. Besides that, it is useful for isolating high-

molecular-weight DNA if lysozyme-digested cells are lysed by the CTAB and where polysaccharide contamination of DNA preparations is a problem. In this protocol, it involves the usage of sodium dodecyl sulfate (SDS) to lyse the cells, rapidly expand and break the chrmosomes (Burden and Whitney, 1995). Proteinase K that include in this method degrade interfering proteins such as histones and nucleases to protect the DNA from excessive degradation. Phenol and chloroform are organic solvents that denature and remove proteins by separating the phase of water and the protein fractions. Addition of chloroform/isoamyl alcohol (CIA) aids in the removal of protein while the DNA is precipitated using isopropanol (Johnson, 1991).

2.2.3 Boiling cell protocol

The boiling cell protocol is an in-house method practiced in many laboratories. It involves the physical lysing of bacterial cell wall by exposing the cell to sudden hot and cold temperature. It is said to be the most cost effective and rapid way to extract DNA as described by Farshad *et al.* (2004). Professor Wilson from Sierra College mentioned that the boiling cell method was successful for Gram negative bacteria as they possess thin cell walls. This protocol does not produce any hazardous organic waste, cheaper and it is also relatively easy to perform. However, the DNA obtained using boiling cell protocol could not be stored for longer period of time in –20°C as degradation will occur (Farshad *et al.*, 2004).

2.2.4 Wizard® Genomic DNA Purification kit (Promega, USA)

The uses of commercial DNA extraction kits readily available from manufacturers have made the DNA extraction process much easier as no proteinase digestion or hazardous organic solvents are necessary (Micka *et al.*, 1996). According to Kephart (1998), the Wizard® Genomic DNA Purification kit (Promega, USA) can be used to isolate genomic DNA from various sources including bacteria, tissue culture cells, animal tissues, plant tissues and yeast. He further added that this DNA extraction kit could be used to study genomic composition of organisms and the expression pattern of target genes of interest. The solutions that were readily produced from this DNA extraction kit such as the Nuclei Lysis Solution aid in the lysis of cells and the nuclei. The presence of RNA could be eliminated by addition of RNase Solution and the purification of DNA could be recovered from impurities such as proteins by adding the Protein Precipitation Solution. Based on the study done by Micka *et al.* (1996), high quality and high yield of DNA can be obtained in less than 45 minutes using Wizard® Genomic DNA Purification kit (Promega, USA). Furthermore, it is safe and rapid as proteinase digestion is absent in this method.

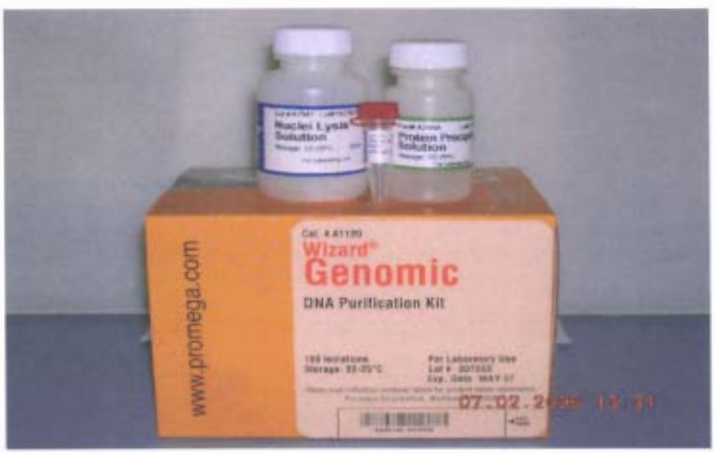

Figure 4. Wizard® Genomic DNA Purification Kit (Promega, USA).

2.3 Spectrophotometry

Nucleic acid concentration in solutions is a fundamental factor for success in molecular biology. Therefore knowledge of its concentration is crucial. Measurement of the absorbance of light by microbial suspensions is the most common method for nucleic acid quantification (Penn, 1991). DNA purity and quantity can be measured using the ultraviolet absorbance spectrophotometer. As described by Brown (1990), the amount of ultraviolet radiation absorbed by a solution of DNA is proportional to the amount of DNA in the sample. This complies with the Beer-Lambert Law of light absorbance over a limited range of cell densities (Penn, 1991). According to Grimont and Grimont (1991), the minimum absorption of DNA is at 260 nm, whereas protein is at 280 nm. Absorbance ratio at 260 nm and 280 nm (A_{260}/A_{280}) of pure DNA is between 1.8 to 2.0. If the value is less than 1.8, the product may be contaminated with either protein or phenol (Brown, 1990). The presence of contaminants such as phenol can influence the concentration and purity (Burden and Whitney, 1995). If the ratio is higher than 2.0 it usually indicate the presence of RNA (Clark and Christopher, 2000).

2.4 DNA amplification

2.4.1 Specific PCR

Polymerase Chain Reaction (PCR) is a process of gene amplification using a thermostable polymerase to produce multiple copies of specific DNA exponentially and rapidly (Towner and Cockayne, 1993). PCR was first established by Karry Mullis in 1985. The PCR involve three major steps which are denaturation, annealing and extension of the target DNA sequence. The amplification occurs through successive cycles of exponential

multiplication from a specific fragment of DNA.

When performing PCR, a few factors can affect its amplification. McOrist *et al.* (2002) found that different extraction methods result in variable sensitivity of PCR detection for DNA of bacterial species. In another study done by Wang *et al.* (1996), primer designs also influence the PCR sensitivity and its efficiency varied significantly. Other parameters such as *Taq* polymerase quality or thermal cycle operation can also affect the sensitivity of PCR reactions (Tyler *et al.*, 1997). The advantages of PCR include simplicity, fast and could also amplify minute amounts of DNA.

2.4.2 Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

The enterobacterial repetitive intergenic consensus (ERIC) analysis PCR involves the use of oligonucleotides targeting short repetitive sequences dispersed throughout various *enterobacteriaceae* genomes (Khan *et al.*, 2002). In ERIC, these small repetitive units of 126 bp contain a conserved central inverted repeat of 40 bp.

ERIC-PCR targets the complete genome and not just one gene's single region. According to Khan *et al.* (2002), ERIC-PCR is currently and widely preferred for typing Gram positive and Gram negative such as *V. parahaemolyticus*, because it is useful for phylogenetic and taxonomical analysis. It has been demonstrated to be the most informative typing method. Compared to other PCR-based analysis, ERIC-PCR offer advantages in terms of simplicity in its technique, rapid to produce reproducible results and less expensive (Leung *et al.*, 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

(i) Sample dilution and bacterial count

a. LB broth (Difco., USA)

b. Peptone water (MERCK, Germany)

c. TPC Agar (OXOID, England)

d. Sterile distilled water

(ii) Phenol/Chloroform/Isoamyl alcohol (PCI) protocol

a. 1X TE Buffer, pH 8.0

b. Proteinase K (Promega, USA)

c. 25% SDS (Bio-Rad, USA)

d. PCI [Phenol/Chloroform/Isoamyl alcohol] (25:24:1)

e. 3 M K-Ac (Hamburg, Germany)

f. Cold Isopropanol (R & M, UK)

g. 70% cold ethanol (Hamburg, Germany)

h. Sterile distilled water

(iii) CTAB protocol

a. 1X TE Buffer

b. 10% SDS (Bio-Rad, USA)

c. 20 mg/ml Proteinase K (Promega, USA)

d. 5 M NaCl (Hamburg, Germany)

e. CTAB/NaCl solution

f. Chloroform/Isoamyl alcohol (24:1)

g. Phenol/Chloroform/Isoamyl alcohol (25:24:1)

h. Isopropanol (R & M, UK)

i. 70% ethanol (Hamburg, Germany)

(iv) Wizard® Genomic DNA Purification kit Cat # A1120 (Promega, USA)

a. Isopropanol (R & M, UK)

b. 70% ethanol (Hamburg, Germany)

c. Nuclei Lysis Solution (Promega, USA)

d. RNase Solution (Promega, USA)

e. Protein Precipitation Solution (Promega, USA)

f. DNA Rehydration Solution (Promega, USA)

(v) Boiling protocol

a. Sterile distilled water

b. Boiling water bath

c. Ice cubes