ISOLATION AND IDENTIFICATION OF *Escherichia coli* FROM RAW VEGETABLES IN KOTA SAMARAHAN AND KUCHING, SARAWAK

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Isolation and Identification of *Escherichia coli* from Raw Vegetables in Kota Samarahan and Kuching, Sarawak

A final year project submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Department of Molecular Biology
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2011
ACKNOWLEDGEMENT

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DECLARATION

I hereby declare that this Final Year Project (FYP) entitled “Isolation and Identification of Escherichia coli from Raw Vegetables in Kota Samarahan and Kuching, Sarawak” is a bonafide and genuine research carried out by me under the guidance and supervision of Dr. Lesley Maurice Bilung and Dr. Micky Vincent, Department of Molecular Biology, Faculty of Resource Science and Technology (FRST), University Malaysia Sarawak (UNIMAS). I also declare that this Final Year Project has not been submitted in any form of another degree or diploma at any university. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references given. FRST shall have the rights to preserve, use and disseminate this FYP report in print or electronic format for academic/research purpose.

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Isolation and Identification of Escherichia coli from Raw Vegetables in Kota Samarahan and Kuching, Sarawak

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ABSTRACT

Escherichia coli is a gram negative and rod-shaped bacterium that is commonly found in the lower intestine of warm blood animals. Almost all E. coli strains are harmless, but some, such as serotype O157:H7 can cause serious food poisoning in humans due to the presence of Shiga-like toxin genes (slt). Eating contaminated raw food is one of the sources of transmission of E. coli. In this study, E. coli was isolated from various raw vegetables in Kuching and Kota Samarahan wet markets and supermarkets in Kuching and Kota Samarahan to determine the number of E. coli occurrences. The five main vegetables were cabbage, cucumber, tomato, winged bean, and carrot. Samples were first inoculated into an enrichment media of Luria Bertani (LB) broth while Eosin-Methylene Blue (EMB) agar was used for isolation. The isolates were tested through a series of biochemical tests. After that, multiplex-PCR assay was carried out to detect the presence of slt and sld genes in the E. coli positive samples. Subsequently, antibiotic susceptibility test for E. coli was determined via disk diffusion technique in determining the antibiotic susceptibility of E. coli isolates against a range of antibiotics. Through a series of biochemical test, 26 (28.89%) isolates were identified as E. coli while none of samples harbored slt and sld genes. All 26 samples were resistance toward erythromycin and sensitive toward nalidixic acid, tetracycline, nitrofurantoin, norfloxacin, and chloramphenicol. Seventeen samples were resistance toward ampicillin while 18 samples were resistance toward carbenicillin.

Keywords: Escherichia coli; raw vegetables; Eosin-Methylene Blue (EMB) agar; Shiga-like toxin (slt), antibiotic susceptibility.

ABSTRAK


Kata kunci: Escherichia coli; sayuran mentah; Eosin-Metilena Biru (EMB) agar; toksik Shiga (slt); kepekaan antibiotik.
TABLE OF CONTENTS

ACKNOWLEDGEMENT I
DECLARATION II
ABSTRACT/ABSTRAK III
TABLE OF CONTENTS IV
LIST OF TABLES VI
LIST OF FIGURES VII
LIST OF ABBREVIATIONS VIII

CHAPTER 1 INTRODUCTION 1
1.1 Introduction 1
1.2 Objectives 3

CHAPTER 2 LITERATURE REVIEW 4
2.1 Escherichia coli 4
2.1.1 General characteristics of E. coli 4
2.1.2 Optimal growth conditions of E. coli 5
2.2 Outbreak of E. coli 5
2.3 Possible source of E. coli: Raw vegetables 6
2.4 Antibiotic susceptibility of E. coli 8
2.5 Multiple antibiotic resistance (MAR) 9
2.6 Detection of E. coli using multiplex PCR method 9
2.6.1 Multiplex Polymerase Chain Reaction (PCR) 9
2.6.2 Shiga Like Toxin (slt) gene 10
2.6.3 Report of multiplex PCR detection of E. coli 10

CHAPTER 3 MATERIALS AND METHODS 12
3.1 Samples collection 12
3.2 Enrichment of bacteria samples 13
3.3 Selective plating and detection of E. coli species on EMB agar 14
3.4 Preparation of stock cultures and working cultures 14
3.5 Escherichia coli identification using biochemical test and Gram staining 15
3.5.1 Indole test, motility test and H2S gas production test 15
3.5.2 Methyl Red test and Voges-Proskauer test 15
3.5.3 Simmon Citrate test 16
3.5.4 Carbohydrate fermentation test 16
3.6 DNA extraction (Boiled cell method) 16
3.7 Multiplex Polymerase Chain Reaction (PCR) 17
3.7.1 Primers used in the study 17
CHAPTER 4

RESULTS

4.1 Presumptive *E. coli* identification in raw vegetables samples
4.2 Gram staining
4.3 Biochemical test result
4.4 Multiplex PCR
4.5 Antibiotic susceptibility test

CHAPTER 5

DISCUSSIONS

5.1 Isolation of *Escherichia coli* on Eosin-Methylene Blue (EMB) agar
5.2 Identification of *Escherichia coli* by using Gram staining
5.3 Biochemical tests
  5.3.1 Indole test
  5.3.2 Methyl Red and Voges-Proskauer tests
  5.3.3 Simmon Citrate test
  5.3.4 Motility test
  5.3.5 Hydrogen sulfide (H2S) production test
  5.3.6 Carbohydrate fermentation test
5.4 Multiplex Polymerase Chain Reaction (PCR)
5.5 The effects of three different locations of wet market and supermarket
5.6 *Escherichia coli* prevalence in vegetables
5.7 Antibiotic susceptibility test of *Escherichia coli*
5.8 Multiple antibiotics resistance (MAR)

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

CHAPTER 7

REFERENCES
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table 3.1:</th>
<th>Location of sampling in Kuching and Kota Samarahan</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.2:</td>
<td>The description of the retail outlets and vegetables that being studied.</td>
<td>13</td>
</tr>
<tr>
<td>Table 3.3:</td>
<td>Type of vegetables tested for the identification of <em>E. coli</em>.</td>
<td>13</td>
</tr>
<tr>
<td>Table 3.4:</td>
<td>Primer sequences for the amplification of slt1 and slt2 genes of <em>E. coli</em>.</td>
<td>17</td>
</tr>
<tr>
<td>Table 3.5:</td>
<td>Specific amount of PCR reagents were used in PCR</td>
<td>18</td>
</tr>
<tr>
<td>Table 3.6:</td>
<td>PCR assays cycling conditions for slt1 and slt2 genes.</td>
<td>19</td>
</tr>
<tr>
<td>Table 4.1:</td>
<td>Number of suspected <em>E. coli</em> occurrences in raw vegetables samples in supermarket and market from three different places.</td>
<td>22</td>
</tr>
<tr>
<td>Table 4.2:</td>
<td>The standard biochemical tests result for identification of <em>E. coli</em>.</td>
<td>24</td>
</tr>
<tr>
<td>Table 4.3:</td>
<td>Biochemical tests results of presumptive <em>E. coli</em> isolates from supermarkets located at Kota Samarahan, Kota Sentosa, and Satok.</td>
<td>24</td>
</tr>
<tr>
<td>Table 4.4:</td>
<td>Biochemical tests results of presumptive <em>E. coli</em> isolates from wet markets located at Kota Samarahan, Kota Sentosa, and Satok.</td>
<td>25</td>
</tr>
<tr>
<td>Table 4.5:</td>
<td>Summary of biochemical tests results of presumptive <em>E. coli</em> vegetables.</td>
<td>26</td>
</tr>
<tr>
<td>Table 4.6:</td>
<td>Frequency of occurrence of <em>E. coli</em> in raw vegetable samples in Kuching and Kota Samarahan.</td>
<td>28</td>
</tr>
<tr>
<td>Table 4.7:</td>
<td>Characteristics of the 26 <em>E. coli</em> strains in the study.</td>
<td>31</td>
</tr>
<tr>
<td>Table 4.8:</td>
<td>Results of the antibiotic resistance test and MAR index for <em>E. coli</em> strains.</td>
<td>32</td>
</tr>
<tr>
<td>Table 4.9:</td>
<td>Percentages of <em>E. coli</em> isolates resistant to various antibiotics.</td>
<td>33</td>
</tr>
<tr>
<td>Table 4.10:</td>
<td>Antibiotic susceptibility pattern of 26 selected strains of <em>Escherichia coli</em>.</td>
<td>33</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 4.1: E. coli colonies (metallic green sheen) as seen on EMB agar 21

Figure 4.2: E. coli cells observed under light microscope using 1000x magnification after Gram staining. 23

Figure 4.3: Simmon citrate test. (a) Positive reaction; (b) Negative reaction. 27

Figure 4.4: Indole test, motility test and H₂S production test. (c) Positive indole test, positive motility test, and negative H₂S production; (d) Positive indole test, negative motility test, and negative H₂S production. 27

Figure 4.5: Figure 4.5: Triple Sugar Iron test: Acid/acid with gas. 27

Figure 4.6: Figure 4.6: (e) Methyl red test; positive reaction. (f) Voges-Proskauer test; negative reaction. 27

Figure 4.7: Percentage of E. coli isolates from raw vegetables samples for the two different markets at three different locations. 29

Figure 4.8: Percentage of E. coli isolates in five different vegetables. 29

Figure 4.9: Agarose gel electrophoresis of the slt₁ (210 bp) and slt₂ (484 bp) gene of E. coli. 30

Figure 4.10: Percentage of antibiotic resistant of E. coli isolated from raw vegetables 34
LIST OF ABBREVIATIONS

°C  Degree Celcius
%  Percent
G  Gram
S  Second(s)
µ  Micro
Rpm  Revolutions per minute
EMB  Eosin Methylene Blue agar
E. coli  Escherichia coli
NA  Nutrient agar
MPN  Most Probable Number
PCR  Polymerase Chain Reaction
FDA  Food and Drug Administration
HUS  Hemolytic-uremic syndrome
IMViC  Indole, methyl red, Voges-Proskauer, and citrate
TSI  Triple sugar iron
slt₁  Shiga like toxin I genes
slt₂  Shiga like toxin II genes
Stx  Shiga toxin
EEC  Enterovirulent E. coli
EHEC  Enterohaemorrhagic E. coli
ETEC  Enterotoxigenic E. coli
EPEC  Enteropathogenic E. coli
EIEC  Enteroinvasive E. coli
EAggEC  Enteroaggregative E. coli
NCCLS  National Committee for Clinical Laboratory Standards
OTA  Organic Trade Association
DNA  Deoxyribonucleic acid
RNA  Ribonucleic acid
STEC  Shiga-Toxin producing E. coli
LB  Luria Bertani
SIM  Sulfide-Indole-Motility
MR  Methyl Red
VP  Voges-Proskauer
EDL  Essential Drug List
ATCC  American Type Culture Collection
MgCl₂  Magnesium chloride
dNTP  Deoxyribonucleotide triphosphate
sdH₂O  Sterile distilled water
mM  Milimolar
Min  Minute(s)
L  Litre(s)
U  Unit(s)
UV  Ultraviolet
bp  Base pair(s)
TBE  Tris/Borate/EDTA
V  Volt(s)
Muller-Hinton
Potential of hydrogen
Hydrogen sulfide
Ferrous sulphate
Ampicillin
Carbenicillin
Erythromycin
Tetracycline
Norfloxacin
Nitrofurantoin
Chloramphenicol
Nalidixic acid
Slant acid/butt acid

Although E. coli (Escherichia coli) is part of normal gastrointestinal flora, some strains which are pathogenic, such as E. coli O157:H7, are known to produce toxins. These include enterohemorrhagic E. coli (EHEC), enterococcal E. coli (EEC) and enterohemorrhagic E. coli (EHEC), which are known to cause "poisoning" or diarrhea even though they usually remain within the intestines by causing localized intestinal inflammation (Hayhurst, 2004).

The National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC), particularly warns of the dangers posed by the rare strain E. coli O157:H7, a pathogenic strain isolated from animals from cattle, sheep, pigs, deer and poultry. This strain causes severe diarrhea, kidney damage and sometimes death. Young children, the elderly,
CHAPTER 1

INTRODUCTION

1.1 Introduction

*E. coli* (*Escherichia coli*) is a bacterium that generally lives in the intestines of animals, including humans (OTA, 2010). This organism is a gram-negative rod-shaped bacterium that is usually found in the lower intestine of warm-blooded organisms (Sliusarenko *et al.*, 2010). In fact, the existence of *E. coli* and other kinds of bacteria within our intestines is essential to aid the human body develop well and to stay healthy. There are about 100 strains of *E. coli*, most of which are beneficial (NYSDOH, 2008).

Although *E. coli* inhabit the intestinal tract as beneficial microorganisms, there also are strains of *E. coli* that are known to produce toxins. There are four to six groups of *E. coli* strains which comprise EEC, EHEC (enterohemorrhagic *E. coli*), ETEC (enterotoxigenic *E. coli*), EPEC (enteropathogenic *E. coli*), EIEC (enteroinvasive *E. coli*), EAEC (enteroadherent *E. coli*), and EAggEC (enteroaggregative *E. coli*) (Marks, 2010). Enterovirulent *E. coli* (EEC) strains cause "poisoning" or diarrhea even though they usually remain within the intestine by producing toxins or intestinal inflammation (Hayhurst, 2004).

The National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC), particularly warns of the dangers posed by the rare strain *E. coli* O157:H7, a pathogenic strain isolated from manure from cattle, sheep, pigs, deer and poultry. This strain can lead to severe diarrhea, kidney damage and sometimes death. Young children, the elderly,
and those with weakened immune systems are the most vulnerable to the *E. coli* O157:H7 infections (CDC, 2010).

In recent years, *E. coli* O157:H7 has been recognized in outbreaks of foodborne illness related to fresh produce (Wallace and Kohatsu, 2008). In October 6, 2006, 199 people were infected resulting in 3 deaths, while 31 suffered hemolytic uremic syndrome after consuming spinach contaminated with the *E. coli* O157:H7 in New Mexico (Grant *et al.*, 2008). In May 2010, an *E. coli* O157:H7 outbreak has infecting at least 19 people in several European countries was traced to the consumption of raw romaine lettuces (Niemira and Cooke, 2010). However, there are no outbreak reports of foodborne disease cause by *E. coli* in Malaysia (Sahilah *et al.*, 2010).

In this study, vegetables are chosen since they are often eaten raw. In Sarawak, several types of vegetables are consumed raw in the popular dish ‘ulam’. Subsequently, eating raw vegetables that is rare or inadequately cleaned is the most common way of possible exposure to *E. coli* contamination (Ingham *et al.*, 2004). The vegetables are bought from either wet markets or supermarkets. In wet markets in Malaysia, a variety of food types including seafood, poultry, meat, and fresh vegetables are commonly sold. Supermarkets normally offer foods under conditions that seem more hygienic than those in wet markets as vegetables are packed neatly before display. Conversely, the hygienic condition and foodstuff handling methods in the packing facilities may be poor thus the occurrence and number of *E. coli* in raw vegetables from both supermarkets and a wet market were determined.

In this study, the isolation of *E. coli* was done by using Luria-Bertani broth as an enrichment media and eosin methylene blue (EMB) agar as a selective agar upon
identification via Gram staining and biochemical tests, multiplex-PCR was performed on the *E. coli* isolates for the detection of Shiga-like toxin I (*slt-I*) and Shiga-like toxin II (*slt-II*) genes. Then, antibiotic resistance test for the *E. coli* isolates was done via the disk diffusion method against a range of antibiotics in determining the antibiotic resistant patterns of the *E. coli* isolates.

### 1.2 Objectives of the study

The aims of the study were:

a) to isolate *E. coli* from vegetables samples obtained from wet market and supermarket in Kota Samarahan, Kota Sentosa and Kuching area.

b) to identify isolated *E. coli* from samples using selective agar as a culture medium (EMB agar), Gram staining technique, and biochemical tests (IMViC and TSI test).

c) to detect *slt-I* and *slt-II* genes of isolated *E. coli* by using multiplex-PCR.

d) to screen the antibiotic susceptibility that can be found in the *E. coli* isolates.
CHAPTER 2

LITERATURE REVIEW

2.1 Escherichia Coli

2.1.1. General characteristics of E. coli

There are five classes of E. coli that are known as the EEC group (enterovirulent E. coli). Each class of EEC is distinctive and dissimilar from the others (Curova et al., 2009). They are enteroinvasive E. coli (EIEC) that invades or passes into the intestinal wall to create severe diarrhea, enterohemorrhagic E. coli (EHEC) which is a type of EHEC, E.coli 0157:H7 that can cause bloody diarrhea and the hemolytic uremic syndrome, enterotoxigenic E. coli (ETEC) that can produces a toxin that acts on the intestinal lining, and is the most common cause of traveler’s diarrhea, enteropathogenic E. coli (EPEC) which can cause diarrhea outbreaks in newborn nurseries, and enteroaggregative E. coli (EAigEC) that cause acute and chronic diarrhea in children (Tucker, 2007).

Spicer (2008) stated that E. coli a member of the Enterobacteriaceae family, which consist of many enteric bacteria, which are facultatively anaerobic Gram-negative that live in the intestinal tracts of animals in health and disease. Its cell wall is composed of a layer of peptidoglycan, opposed to the phospholipid bilayer of gram-positive bacteria. It is rod shaped bacteria that uses flagella for motility. It can grow in the presence or absence of oxygen. Most E. coli strains are harmless except the EEC which can cause serious food poisoning in humans. This bacterium can be cultured easily and its genetics are fairly simple and easily
Manipulated or duplicated through a process called mutagenic (Young et al., 2010). Both pathogenic and non-pathogenic strains of *E. coli* can be isolated from contaminated vegetables, feces, infected animals (cattle), and mainly found in guts (Heaton and Jones, 2007).

2.1.2 Optimal growth conditions of *E. coli*

The optimum temperature for *E. coli* to grow is 37°C which explains the reason why *E. coli* can be found in the guts of mammalians. *E. coli* appears to be more tolerant of low pH than of high pH which is approximately pH 7.0 and can grow in any low to medium concentration of salt. *E. coli* growth is also affected by the presence of glucose. *E. coli* grows in environment with no glucose, however survival rate is low (Don, 2008). In the presence of higher glucose concentrations, *E. coli* would absorb the extra glucose and convert it into energy, allowing all its chemical process to proceed faster and with a more rapid growth and reproduction rate (Negrete et al., 2010).

2.2 Outbreak of *E. coli*

In 2006, an outbreak of *E. coli* O157:H7 caused by consumption of contaminated fresh spinach occurred in United States. One hundred and ninety nine persons infected with the outbreak strain of *E. coli* O157:H7 have been reported to CDC from 26 states. Three deaths in confirmed cases have been associated with the outbreak (Kulasekara, et al., 2006).
In July 2008, two children experienced *E. coli* O157-associated hemolytic uremic syndrome (HUS) after consuming raw milk purchased at a retail market and a farm located in Connecticut. Guh *et al.* (2010) identified raw milk consumption was associated with illness because *E. coli* O157: NM outbreak strains were identified in stool specimens of six case patients and one milking cow.

The multistate outbreak of human *E. coli* O145 which is referred as Shiga toxin-producing *E. coli* or STEC infections linked to shredded romaine lettuce from a single processing facility occurred in United States in 2010. Among the 30 patients with available information of infection of this bacterium, 40% were hospitalized. Three patients have developed a type of kidney failure known as hemolytic uremic syndrome, or HUS. There are no deaths reported in the incident (CDC, 2010). In Malaysia, there are no outbreak reports of foodborne disease cause by *E. coli* O157:H7. However, these serotypes have been isolated from clinical samples as well as beef samples (Son *et al.*, 1998).

### 2.3 Possible source of *E. coli*: Raw vegetables

*E. coli* was first recognized as a foodborne pathogen in 1982 and the definite number that occurred is possibly much bigger due to *E. coli* infections did not develop a reportable disease until 1987 (Rangel *et al.*, 2005). As a result, Central of Disease Control (CDC) states that only the most geographically focused outbreaks would have gathered plenty attention in order to prompt further investigation. Fresh vegetables once are thought to be quite free of disease-producing pathogens. Lately, however, outbreaks of food-borne illness linked vegetables have become more common (Soderstom *et al.*, 2008).
According to Chai et al. (2008) research, vegetables in Malaysia which are mostly 'ulam' containing various pathogenic microbes including gram negative E. coli. In addition, on September 14, 2006, the Centers for Disease Control (CDC) informed the FDA that E. coli had connected the spinach to several illnesses (Kulasekara et al., 2006). Furthermore, some vegetables are dirtied with animal (especially cattle) manure which comprises a lot of E. coli. Some farmers use manure as an excellent fertilizer and soil conditioner for their crops. Additional sources that have been identified as sources of contamination include unpasteurized apple juice and cider (Sapers et al., 2009), orange juice, alfalfa and radish sprouts (Neeto et al., 2009), lettuce, spinach, and water (Doering et al., 2009). Raw or improperly composted manure, irrigation water containing untreated sewage or manure, and contaminated wash water are the cause of E. coli presence in vegetables. Contact with mammals, reptiles, fowl, insects and unpasteurized animal products are other sources of contamination (Davis and Kandell, 2005).

Variations in microorganisms have undeniably contributed to foodborne disease, as have changes in growing, harvesting, distribution, processing and consumption practices (Delaquais et al., 2007). Unclean surfaces, including human hands that come in contact with whole or cut produce, represent possible points of cross-contamination through the food system such as growing, harvesting, packing, processing, shipping and preparing produce for consumption (Galvez et al., 2009; Ching et al., 2008). According to Chai et al. (2007), raw vegetables from supermarkets were twice the prevalence of Campylobacter spp. in vegetables from wet markets in Malaysia. It can be hypothetically assume that the existence of E. coli may be higher in vegetables in the supermarket compare to the wet market in Malaysia.
2.4 Antibiotic susceptibility of *E. coli*

The change of *E. coli* resistance to certain antimicrobial agents is always linked to exposure of bacteria to antibiotics obtainable in the environment (Gaze *et al.*, 2008). The spread of antibiotic resistance genes are because of mutations, transposition of the bacterial genome and genetic exchange between bacteria (Chai *et al.*, 2008). *E. coli* has also been known to have the ability to exchange genetic information with other organisms gaining some of that organism’s characteristics. The *E. coli* 0157:H7 is an example of this action. *E. coli* 0157:H7 was infected with a bacterial virus and that particular virus had the ability to insert its own DNA into the bacteria’s chromosome without harming the bacterium (Juneja and Sofos, 2010).

*E. coli* usually was not affected by either penicillin or ampicillin in antibiotic resistance condition. This is because *E. coli* is a gram-negative bacterium and produces β-lactamase, an enzyme that breaks down the β-lactam rings of the penicillin. So, it is able to avoid the β-lactams from the penicillin from destroying its cell membrane due to the presence of β-lactamase (Don, 2008).

Conversely, chloramphenicol, tetracycline and streptomycin were all able to kill the *E. coli* bacteria because these three antibiotics inhibit the activity of enzymes that are involved in DNA and RNA synthesis (Don, 2008). *E. coli* has β-lactamases and hence cannot be killed by β-lactam antibiotics. Also, there was folate in the nutrient broth so the sulphonamides had no effect on the *E. coli*, and the best way to kill *E. coli* is to treat it with antibiotics that attack the DNA and RNA synthesizing enzymes (Skold, 2010). To improve the research, several of antibiotics with different mechanism can be used such as chloramphenicol, erythromycin, ciprofloxacin, tetracycline, and others (Don, 2008).
2.5 Multiple antibiotic resistance (MAR)

Multiple antibiotic resistance (MAR) is a condition of organisms causing disease to resist distinct antibiotics of a wide variety (Bushman, 2002). Numerous microorganisms have survived for thousands of years by being able to adapt to antimicrobial agents. They do so through spontaneous mutation or by DNA transfer. This process allows some bacteria to against the assault of particular antibiotics and subsequently rendering the antibiotics ineffectively (Guminski et al., 2002). These microorganisms apply several mechanisms in attaining multiple antibiotics resistance such as no longer depending on a glycoprotein cell wall, enzymatic deactivation of antibiotics, and decreased cell wall permeability to antibiotics. From this process, MAR organisms can be formed and their existence in the environment threatened the human health (Stix, 2006).

2.6 Detection of *E. coli* using multiplex PCR method

2.6.1. Multiplex Polymerase Chain Reaction (PCR)

Multiplex-PCR is a modification of PCR that amplifies genomic DNA samples using multiple primers. Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information can be obtained from a single test run. Otherwise, it would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized in order to work correctly within a single reaction and amplicons sizes should be different enough to create distinct bands when visualized by gel electrophoresis (Pestana et al., 2010). Multiplex PCR is a popular method
and has been used widely for the detection of *E. coli* O157:H7 in previous studies through the detection of Shiga like toxin genes (Radu et al., 2001; Yazdi et al., 2011).

### 2.6.2 Shiga Like Toxin (SLT) gene

Shiga-like toxin, also known as verotoxin, is a toxin generated by *E. coli* and named for its likeness to the AB5-type Shiga toxin produced by the bacteria *Shigella dysenteriae*. There are two types, known as slt₁ and slt₂. Ontario rediscovered the Shiga toxin produced by *Shigella dysenteriae* in *E. coli* in 1977 (Werber et al., 2008).

The *E. coli* shiga toxin version was named "verotoxin" due to the ability of the toxic protein to kill Vero cells in culture. Shortly after, this verotoxin was denoted to as Shiga-like toxin because of its similarities to Shiga toxin. It requires highly specific receptors on the cells' surface in order to attach and enter the cell. Species such as cattle, swine, and deer which do not carry these receptors might harbor toxigenic bacteria without slightly ill effect, shedding them in their feces, from where they possibly will be spread to humans (Gabius, 2009).

### 2.6.3 Report of multiplex PCR detection of *E. coli*

In a previous study, Kumar et al. (2008) studied that Shigella-toxigenic *E. coli* (STEC) is prevalent in various seafoods such as fresh fish, clams and water in India and non-O157 serotype is more common. Koitabashi et al. (2006) proved that *E. coli* O157 strains from marketed beef in Malaysia carried Thai-12 q-stx₂ sequence which formed little or no stx₂ were
detected by using the PCR assay. Therefore, 30% of stx2-positive *E. coli* O157 strains from those beef samples produced slightly or no stx2 toxin. In Lleo *et al.* (2005) research, quantification by PCR using slt1 primers helped in detection non-culturable cells present in water samples in the different zones of Italy and only four samples were positive for *E. coli* in this research. Khandaghi *et al.* (2010) obtained a total of 282 samples of soil and vegetables (lettuce, cabbage, carrot and radish sprout), from manure fertilized agriculture farms in Tabriz city, Iran and tested by using multiplex PCR to confirmed *E. coli* O157:H7 isolates. As a result, five samples of soils (1.77 %) and one sample of vegetable (0.35 %) of total samples from this location contaminated with *E. coli* O157:H7.
CHAPTER 3

MATERIALS AND METHODS

3.1 Samples collection

Raw vegetables were purchased from three wet markets and three supermarkets in Kuching, Sarawak as shown in Table 3.1.

Table 3.1: Location of sampling in Kuching and Kota Samarahan, Sarawak.

<table>
<thead>
<tr>
<th>Target place</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supermarket I</td>
<td>Kota Sentosa</td>
</tr>
<tr>
<td>Supermarket II</td>
<td>Kota Samarahan</td>
</tr>
<tr>
<td>Supermarket III</td>
<td>Satok</td>
</tr>
<tr>
<td>Wet market I</td>
<td>Kota Sentosa</td>
</tr>
<tr>
<td>Wet market II</td>
<td>Kota Samarahan</td>
</tr>
<tr>
<td>Wet market III</td>
<td>Satok</td>
</tr>
</tbody>
</table>

Sampling was done on a weekly basis from October 4, 2010 to January 24, 2011. At each market and supermarket, one stall and one shop were picked randomly. The descriptions of both supermarket and wet market are shown in Table 3.2. Table 3.3 showed the differences between the conditions in the supermarkets and wet markets from the retail outlets in general. Purchased vegetables were packed separately to avoid cross contamination and kept in the polystyrene box containing ice packs and transported to the laboratory to be processed immediately. In this research, five types of raw vegetables were used in this experiment as shown in Table 3.3.
Table 3.2: The description of the retail outlets and vegetables that being studied.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Wet market</th>
<th>Supermarket</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Open</td>
<td>Enclosed</td>
</tr>
<tr>
<td>Appearance</td>
<td>Clean</td>
<td>Clean</td>
</tr>
<tr>
<td>Vegetables freshness</td>
<td>Fresh</td>
<td>Fresh</td>
</tr>
<tr>
<td>Packaging</td>
<td>No packaging</td>
<td>Vegetables on styrofoam over-wrapped with polyethylene film</td>
</tr>
<tr>
<td>Display duration</td>
<td>&gt; 6 hours</td>
<td>2-3 days</td>
</tr>
<tr>
<td>Display condition</td>
<td>At ambient temperature on chilled stall table.</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: Type of vegetables tested for the identification of *E. coli*.

<table>
<thead>
<tr>
<th>English name</th>
<th>Local name</th>
<th>Scientific Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winged Bean</td>
<td>Kacang Botol</td>
<td><em>Psophocarpus tetragonolobus</em></td>
</tr>
<tr>
<td>Tomato</td>
<td>Tomato</td>
<td><em>Solanum lycopersicum</em></td>
</tr>
<tr>
<td>Cucumber</td>
<td>Timun</td>
<td><em>Cucumis sativus</em></td>
</tr>
<tr>
<td>Carrot</td>
<td>Karot</td>
<td><em>Daucus carota</em></td>
</tr>
<tr>
<td>Cabbage</td>
<td>Kubis</td>
<td><em>Brassica oleracea</em></td>
</tr>
</tbody>
</table>

3.2 Enrichment of bacteria samples

Each of the vegetables was cut into small pieces, and then a 25 g of portion was weighed aseptically and added into stomacher bags. The cut sample was crushed into very small pieces of sample and added 225 ml of Luria-Bertanni (LB) broth (Sigma, USA) individually for each