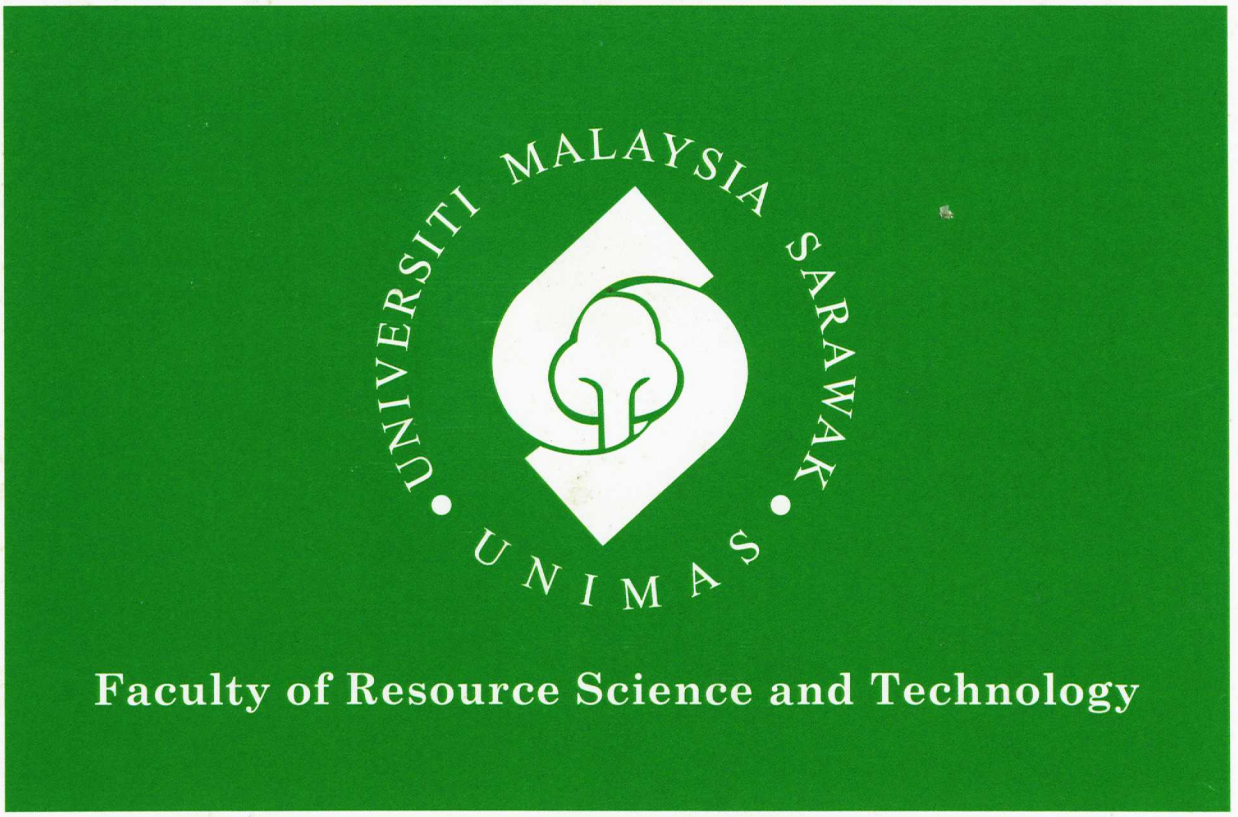

Enumeration of *Escherichia coli* O157:H7 in drinks from different food premises in Kota Samarahan

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Bachelor of Science with Honours
(Resource Biotechnology)
2018

UNIVERSITI MALAYSIA SARAWAK

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Final Year Project Report ☑

Masters ☐

PhD ☐

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Enumeration of *Escherichia coli* O157:H7 in drinks from different food premises in Kota Samarahan

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ABSTRACT

Contamination of drinks with *E.coli* O157:H7 served in food premises such as restaurants can cause haemorrhagic colitis and haemolytic uremic syndrome to humans. The presence or absence of faecal pathogen was demonstrated using coliform group as indicator microorganisms. Therefore, this study was conducted to detect the presence *E.coli* O157:H7 in drinking water from restaurant to ensure safe and potable drinking water is served to the consumer. A total of 30 drinks samples including six samples of cold plain water, six samples of iced tea, and six samples of iced milo, six samples of syrup and six samples of iced milk tea were bought from six restaurants in Kota Samarahan. Most Probable Number (MPN) procedure was used in this study to enumerate the MPN values of coliform bacteria in each drinks collected. The presence of *stx1* gene produced by *E.coli* O157:H7 in water samples were identified by using PCR assay. There is one (3.33%) out of 30 samples were positive carried *stx*1 gene. This study showed that one of the drinks collected from restaurants was contaminated with faecal contamination which was not safe to drink by the customers.

Key words: *E.coli, E.coli* O157:H7, food premises, MPN method, PCR assay

ABSTRAK

Pencemaran minuman dengan *E.coli* O157:H7 yang dihidangkan di premis makanan seperti restoran boleh menyebabkan kolitis berdarah dan haemolytic uremic syndrome kepada manusia. Kewujudan atau ketiadaan patogen najis ditunjukkan dengan menggunakan kumpulan koliform sebagai penunjuk mikroorganisma. Oleh itu, kajian ini dijalankan bagi mengesan kehadiran *E.coli* O157:H7 dalam air minuman dari restoran untuk memastikan air minuman yang boleh diminum dan selamat diminum oleh pengguna. Sebanyak 30 sampel minuman termasuk enam sampel air sejuk kosong, enam sampel teh ais, enam sampel milo ais, enam sampel sirap dan enam sampel teh susu ais yang dibeli dari enam restoran di Kota Samarahan. Kaedah *Most Probable Number* (MPN) digunakan dalam kajian ini untuk menghitung nilai MPN bakteria koliform dalam setiap minuman yang dikumpul. Kehadiran gen *stx1* yang dihasilkan oleh *E.coli* O157:H7 dalam sampel air telah dikenal pasti dengan menggunakan ujian PCR. Terdapat satu (3.33%) daripada 30 sampel yang positif membawa gen stx1. Kajian ini menunjukkan bahawa salah satu minuman yang dibeli dari restoran tercemar dengan pencemaran najis yang tidak selamat diminum oleh para pelanggan.

Kata kunci: *E.coli*, *E.coli* O157: H7, premis makanan, kaedah MPN, ujian PCR

TABLE OF CONTENT

Declaration	i
Acknowledgements	iii
Abstract/ *Abstrak*	iv
Table of Content	v
List of Tables	vi
List of Figures	vii
List of Abbreviations	viii
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW	
2.1 Waterborne disease outbreaks	3
2.2 Drinking water quality	4
2.3 Most Probable Number	5
2.4 Indicator microorganisms	5
2.4.1 Total coliform bacteria	6
2.4.2 Faecal coliform	7
2.4.3 *E.coli*	7
2.5 *Escherichia coli* O157:H7	8
2.6 Disease caused by *E.coli* O157:H7	8
2.7 Foodborne outbreak associated with *E.coli* O157:H7	9
2.8 Outbreak in food premises	10
CHAPTER 3: MATERIALS AND METHODS	
3.1 Materials	11
3.2 Methods	
3.2.1 Samples collection	11
3.3 Detection of faecal coliform, i.e *E.coli*	
3.3.1 Most Probable Number (MPN)	11
3.3.2 Gram staining	12
3.4 Molecular analysis	
3.4.1 Genomic DNA extraction	13
3.4.2 Polymerase Chain Reaction (PCR)	13
3.4.3 Agarose Gel Electrophoresis (AGE)	15
CHAPTER 4: RESULTS	
4.1 Detection of faecal coliform	16
4.2 Isolation and identification of *E.coli*	17
4.3 Amplifacation of *stx1* gene using PCR	19
CHAPTER 5: DISCUSSION	
5.1 Detection of faecal coliform in drinks collected from restaurants	20
5.2 Amplification of *stx1* gene using PCR assay	22
CHAPTER 6: CONCLUSION	24
CHAPTER 7: REFERENCES	25
CHAPTER 8: APPENDICES	35

LIST OF TABLES

Tables		Pages
Table 3.1	Master mix component for PCR	18
Table 3.2	Characteristic of primers to detect *stx1* gene *E.coli* O157:H7	18
Table 3.3	PCR condition for *E.coli* O157:H7	18
Table 4	Gram staining result from each positive Completed Test	23

LIST OF FIGURES

Figures		Pages
Figure 1	The bar graph represent the average MPN values/ 100 ml of drinks samples recorded six restaurants	20
Figure 2	Green metallic sheen colonies produced on EMB agar	21
Figure 3	Gram staining result shows Gram negative, rod shape and non-spore forming bacteria under Oil Immersion lens 100 x	22
Figure 4	The bands of PCR products for detection of *stx1* gene	24

LIST OF ABBREVIATIONS

AGE	Agarose gel electrophoresis
BGLB	Brilliant green lactose broth
DNA	Deoxyribonucleic acid
EMBA	Eosine methylene blue agar
LTB	Lauryl tryptose broth
MPN	Most probable number
mM	miliMolar
mL	miliLitre
µl	Microlitre
NB	Nutrient broth
Rpm	Revolution per minute
TBE	Tris-borate -EDTA
UV	Ultraviolet
WHO	World Health Organization
WQI	Water Quality Index
^{0}C	Degree Celcius

1.0 INTRODUCTION

Consumption of contaminated drinking water might be linked to severe life-threatening disease also known as waterborne diseases. Foodborne and waterborne diseases always become a major problem in developing countries compared to developed countries (Portier *et al.*, 2013). According to World Health Organization (WHO) (2002), contaminated drinking water may become a major burden to human health as it can contribute to waterborne disease. There are many types of popular waterborne outbreak agent that can survive in water including drinking water which can harm human health such as bacterial agents, viral agents, pathogenic agents and also chemical agents (Leclerc *et al.*, 2002). Other than consuming water, this pathogenic agent can be transmitted from directly dermal contact with water.

Bacterial pathogens that are popular in causing waterborne disease such as diarrheal illness, typhoid fever, cholera, cryptosporidium, giardiasis and legionellosis which are causes by *Escherichia coli*, *Salmonella typhi*, *Vibrio cholera*, *Cryptosporodium parvum*, *Giardia lamblia* and *Legionella pneumophila*. It has been reported that between 2011 and 2016 there are 381 cases associated with protozoan disease worldwide (Efstratiou *et al.*, 2017) 7% from 6939 deaths in the United States were associated with several types of pathogens including *E.coli* transmitted by the fecal-oral route (Gargano *et al.*, 2017). *E.coli* is a member of fecal coliform group and commonly used as an indicator to monitor the microbiological quality in drinking. Their presence in drinking water indicates recent fecal contamination which means potential presence of enteric pathogens (Figueras & Borrego, 2010).

Hygiene standards of food premises that sold, handled, prepared, packaged, served, process and stored food and drinks or eating places such as restaurants and stalls may reflect the quality of food and drinks served to the customers. However, restaurants and stalls with low sanitary conditions may contribute to foodborne and waterborne outbreak because once the workers make mistakes, it may affect many people. Golan *et al.* (2004) reported that 700 people had contracted a foodborne illness and 4 children died after eating contaminated meat from restaurant, five diners experienced diarrhoea and vomiting after eating raw oysters (Baker, 2010).

As water is also used in food preparation and making drinks for customer, it is very crucial to abide the guidelines of drinking water quality standards established by Ministry of Health Malaysia. The maximum acceptable value for total coliform and *E.coli* is zero in 100 ml (Ministry of Health Malaysia, 2006). This study is important in terms of gauging the level of bacteria presence in drinking water with *E.coli* as well as determining the quality and safety of the drinking water served from restaurant in Kota Samarahan. The occurrences of some pathogenic bacteria in drinking water may increase the risks of water related diseases and health problems in local residents.

The objectives of this study are:

1. To enumerate and isolate the total coliform bacteria in drinks from different food premises in Kota Samarahan.
2. To detect the presence of *stx1* gene of isolated *E.coli* from drinks by using Polymerase Chain Reaction (PCR).

2.0 LITERATURE REVIEW

2.1 Waterborne disease outbreaks

Waterborne disease is described the complex interaction including social, biological environmental process with pathogen that are harmful to human which then resulted to an infectious individual (Koopmand and Logini, 1994). One of the regions in United States, Montana is reported to have the largest waterborne outbreak spread through rainfall (Weniger *et al.*, 1983). Furthermore, there are many types of pathogen that can cause waterborne disease which can be transmitted to the people which having contact or consume the infected water. The most common of infectious agents that cause waterborne disease including viruses, bacteria and parasites such as *Shigella, Vibrio cholera, E.coli,* coliform bacteria, poliovirus, *Giarda lamblia* and *Cryptosporidium parvum*.

Diarrhea is one of the major cases that are usually reported to cause waterborne disease which caused by coliform bacteria, *Escherichia coli* O157:H7 (Swerdlow *et al.,* 1992). The infected patients will suffer from malaise, anorexia, abdominal cramp and may lead to bloody diarrhea. Usually this type of disease is more common to be infected by young and elderly people. Other than that, giardiasis is also a known waterborne disease which is caused by *G. lamblia* and usually associated with drinking water. It can lead to various complications such as weight loss and dehydration from diarrhea.

2.2 Drinking water quality

Water quality standards is established in every country in this world to monitor and control the quality of water in terms of chemical, physical and biological aspects to ensure the quality of waters is safe to be used for the public (Radojević *et al.*, 2012). In addition, Malaysia's government also implemented the National Drinking Water Quality Surveillance Programme (NDWQSP) to standardize a potable and safe drinking water provided by the producers to the people. The recommended value of total coliform bacteria and *E.coli* in raw quality water is 5000 MPN per 100 ml while zero per 100 ml in drinking water. The indicator parameters such as residual chlorine and faecal coliform are responsible to rate the water quality in Malaysia which can cause many waterborne diseases to the public if they are presence in high level.

Water management and treatment is important to distribute safe and potable water based to the public. In Kuching, Kuching Water Board (KWB) is responsible to supply safe and potable water based to Kuching city areas. Before the treated water is being supplied to the surrounding area, Public Works Department of Sarawak will make sure whether the water is in good quality or not.

2.3 Most Probable Number (MPN) method

The MPN procedure involves a multiple tube fermentation technique where three or more decimal dilutions of the sample are inoculated into tubes of broth medium and incubated at a specific temperature and for a specific time (Pavic *et al.*, 2010). Firstly, MPN method will determine the presence of coliforms in the tubes, and after that the faecal coliforms will be detected, and the last phase is to confirm whether *E. coli* is present. The presence of the gas in every each positive tube will be calculated with the most probable number present can be estimated from a standard statistical MPN table (Kinzelman *et al.*, 2003).

This method has been shown to produce satisfactory results with naturally-contaminated and artificially-contaminated water in sealed containers (including mineral and spring water) and pre-packaged ice. The presence of coliforms, faecal coliforms and aerogenic *E. coli* in water may be determined by means of the MPN procedure.

2.4 Indicator microorganisms

Indicator microorganisms are the type of bacteria that is used to detect the any presence of potential disease pathogens in food and water. They must be non-pathogenic, have similar survival rate with pathogens and can be detected at low concentration on water. A sample taken from water is considered contaminated if high number of indicator microorganisms detected. Some criteria that are assigned with indicator microorganisms are the bacteria must be present whenever enteric pathogen are present and can be easily isolated and amplified through simple experimental methods. Other than that, indicator microorganisms can give benefit

to the people as their presence in food or water is safe to be consumed or not. Coliform bacteria and *E.coli* are the most common bacteria used as indicator microorganism because it can indicate various pathogens associated with them in the water samples (Khan *et al.,* 2013). However, there is limitation to test water with indicator microorganisms as it is only limit for bacteria and viral but no indicator protozoa have been stated (Mara and Horan, 2003)

2.4.1 Total Coliform bacteria

Total coliforms are frequently found in soil, vegetation and in surface water and are generally harmless. They are members of *Enterobacteriaceae* family which are facultatively anaerobic, Gram-negative, non-spore forming, oxidase-negative, saprophytic but potentially pathogenic, rod-shape that utilize lactose to produce acid and gas within 48h at 35^{0}C via multiple-tube fermentation technique. Next, their characteristics are grouped based on β-galactosidase postitive reactions which gives researchers to give a confirmation for lactose fermentation while using multiple tube fermentation.

They are most prevalence in the intestine of warm-blooded animals and in faeces but are also common in water, milk and soil. In addition, MacConkey (1909) classified coliform bacteria into for sub-groups according to their sucrose and dulcitol reactions. In his study of the classification of coliform bacteria, Malcolm (1938) explained that the presence of total coliforms in water indicates faecal contamination as evidence of sewage pollution which frequently occur in processing and milk storage. They are also used as indicator microorganisms to evaluate the water quality in recreational and drinking water by using Most Probable Number technique, Membrane filtration method and also Colilert system.

The presence of this type of bacteria in 100ml of drinking water will cause contamination of the water (Savichtcheva and Okabe, 2006).

2.4.2 Faecal coliform

Faecal coliform bacteria or thermo tolerant bacteria are come from a sub-set of total coliform group. They also possess the same definition with total coliform but they lived at 44.5°C in the gastrointestinal tract of warm-blooded animals. Ishii and Sadowsky (2008) stated that their presence also indicate faecal contamination as they should not grow in water.

2.4.3 *E.coli*

Most of *E.coli* strains are harmless but some of them are pathogenic, such as EHEC can cause severe disease to humans and animals (Clements *et al.*, 2012). *E.coli* is an example of single species of fecal coliform that are identified in 1885 by Theodor Escherich and the best biological drinking water indicator (Edberg *et al.*, 2000). *E.coli* is a member of the family Enterobateriaceae which includes many genera such as *Salmonella, Shigella* and *Yersinia. E.coli*, bacteria is found in the intestine of warm-blooded animal which gives benefit to maintain the physiology of the healthy host. Therefore, the presence of *E.coli* in water or food will indicate the sewage or animal waste contamination. *E.coli* may be washed into the rivers, streams, lakes or groundwater during rainfall which then be used as drinking water sources without any treatment may cause disease to the public. However, most of the *E.coli* is harmless but *E.coli* O157:H7 strain can cause serious illness such as diarrhea.

2.5 *Escherichia coli* O157:H7

Enterohemorrhagic *E.coli* is known as pathogenic *E.coli* and *E.coli* O157:H7 is one of thousands of serotypes of *E.coli*. This strain can produce Shiga toxins (*stx*) and cause hemorrhagic colitis (HC) and can lead to haemolytic uremic syndrome (HUS) in humans (Lim *et al.*, 2010). Most of the *E.coli* strains are useful and harmful to human. However, there are some strains that have acquired virulence factors through plasmids, transposons, bacteriophages which then become evolved into pathogenic *E.coli*. It is named *E.coli* O157:H7 due to the expression of somatic (0) antigen 157 and flagella (H) antigen 7.

A total of 99% identical strains to the virulence-associated genes are able to survive in numerous environments such as soil, water, food and animal reservoirs. It can survive in 50 °C for six days and for a long time in at cold temperature in water (Wang and Doyle 1998). Furthermore, Ryu and Beuchat (2005) concluded that *E.coli* O157:H7 were resistance to chlorine that are normally used in food industry which are responsible in the foodborne outbreak associated with this organism.

2.6 Disease caused by *E.coli* O157:H7

The gene that is produced by *E.coli* O157:H7 which is shiga like toxin gene can cause severe, acute haemorrhagic diarrhea and abdonminal cramps to human. The persons that are exposed to this organism require only one to three days getting the symptoms of bloody diarrhea. If the infected person becomes ill in longer than three days, the disease can progress to haemolytic uremic syndrome (HUS) (Banatvala *et al.*, 2001). HUS is one of the clinical syndromes characterized by progressive kidney failure, low platelet count and most commonly cause acute

kidney injury in children. The possibility of 10% infected persons with HUS to be subjected into death is ranging from 3% to 5% which are mostly occur in young children (Grisaru, 2014). Other symptoms of disease caused by this organism include abdominal cramps, fever and vomiting.

2.7 Foodborne outbreak associated with *E.coli* O157:H7

Other than that, *E.coli* O157:H7 also become a major foodborne pathogen causing disease as it can be transmitted to humans through bovine food products and fresh produce such as lettuce are the most common source for foodborne outbreaks. Undercooked or unpasteurized animal products such as ground beef and unpasteurized milk and cheese are often associated with foodborne outbreaks as (Ackers *et al.*, 1998; Cassin *et al.*, 1998; Kassenborg *et al.*, 2004; Lynch *et al.*, 2009; Centres for Disease Control and Prevention, 2012). The latest outbreak associated with *E.coli* O157:H7 reported in May 2018, six people consumed romain lettuce have been hospitalized including one person who developed haemolytic uremic syndrome but no death reported (CDC, 2018). Majority the transmission of *E.coli* O157:H7 to human is through consumption of contaminated food and water (Rangel *et al.*, 2005). However, this organism can be killed by cooking food and boil the water to a minimum temperature of 71 ^{0}C before consumption.

2.8 Outbreaks in food premises

Food premises means a floor area where food or drink intended for public consumption is sold, supplied, handled, prepared, packaged, displayed, served, stored, transported or dispensed. Restaurants, hotels, cafes, shops, supermarkets, school canteen, warehouse as well as stalls are the type of food premises which can be classified into four classes based on the Food Act 1984. Every food premises need a license activity as defined in Food Safety and Quality Act 2001. Every operator of food premises must ensure that all food and water in the premises is protected from contamination as good hygiene practice and maintained a sanitary condition in the premises must be applied during food handling and preparation (Newbold *et al.*, 2008).

There are some cases of gastrointestinal disease caused by Norovirus that are reported in 2009 which involved 240 persons who attended a restaurant in England (Smith *et al.*, 2012). In 2008, sandwiches sold that prepared by infected employees of Vietnamese restaurant in United States were contaminated with *Salmonella enteritidis* (Hedican *et al.*, 2009). Greig *et al.* (2007) stated that the transmission of foodborne agents can be transmitted from infected worker to the food. Proper food handling and preparation with good sanitation is important in food services as well as reducing the contamination of the food and water with foodborne pathogen. In Malaysia, food poisoning cases occur in schools is increasing throughout the year until 2015. There is a case involving 130 students in Penang were affected with food poisoning. Prevalence of *E.coli* O157:H7 in food in Malaysia were reported at 2.5% in cooked foods from school canteen, (76.6%) in ready to eat food and (54.54%) in beef and beef products (Chang *et al.*, 2013; Elexson *et al.*, 2017; Premarathne *et al.*, 2017).

2.0 MATERIALS AND METHODS

2.1 Materials

Agarose powder, Crystal violet, Eosin Methylene Blue (EMB), Glycerol stock, Iodine, agar, Lauryl Tryptose Broth, Luria-Bertani (LB) broth, Nutrient Agar, Safranin, Tris-borate-EDTA buffer, 70% ethanol solution.

2.2 Methods

3.2.1 Samples Collection

A total of 30drinks with five flavours (iced tea, iced milk tea, syrup, iced milo and plain cold water) of each restaurant were purchased from Dapur Mak Wa, Sahira Corner, Penang Bistro, Café Station 9 and Lee Young Cafe Kedai Aunty in Kota Samarahan. Then, the samples were labelled and placed on ice inside ice box and immediately stored at 4^{0}C for 2-4 hours in the fridge before starting the experiment.

3.3 Detection of faecal coliform, i.e *E.coli*

3.3.1 Most Probable Number (MPN)

The most probable number (MPN) procedure is used widely to estimate microbial densities in many matrices including foods and water. The method of Most Probable Number (MPN) was used based on the protocol provided by Feng *et al.* (2013). For the presumptive test, lauryl tryptose broth (LTB) fermentation tubes were used to screen the presence of coliform organisms by observing the gas production within Durham tube. In this steps, nine of test tube containing sterile LTB broth incorporated with Durham tube in each tube, 3 test tubes containing 10 of double strength LTB with 10 mL portions of drinking water samples, 3 of each single strength LTB with 1 mL portions of drinking water samples and 3 of each

single strength of LTB with 0.1 mL portions of drinking water are inoculated. All these water samples were carried out aseptically by using sterile pipettes. Then, the water samples were incubated at 37 °C and observed for the growth and gas production after 48 hours. The MPN per gram for each positive tubes were recorded with the three-tube MPN table available in Appendix 2 of Bacteriological Analytical Manual, Blodgett (2010).

From each of positive tubes of LTB in presumptive test, a loopful was streaked onto Eosin Methylene Blue (EMB) in completed test. Then, the EMB agars were incubated for 24 hours at 37 °C. Next, coliforms bacteria produced small colonies with dark centre while *E.coli* produced shiny metallic green colonies on the EMB agar as shown in Figure 2. The shiny metallic green colonies were isolated and stroke on the nutrient agar and incubated for 24 hours at 37°C.

3.3.2 Gram staining

Gram staining was carried out based on the protocol provided by Barile (2012). A single of actively growing bacteria from each nutrient agar plate was transferred onto microscope slide and spread evenly to produce a thin film. A drop of distilled water was added to the slide and dried up the water by rapidly passes the slides over the flame of Bunsen burner to stain the smear. Next, crystal violet was added on the slide and left for one minute and washed with distilled water. The specimen was covered with iodide solution for one minute and washed again with distilled water.

For decolourization step, a stream of 90 % of ethanol was run on the microscope slide until the blue staining colour no longer comes off and rinsed with distilled water immediately. Next, the specimen was gently flooded with Safranin and let it stand for 45 seconds. The slide was rinsed with distilled water and blotted dry with soft tissue and then will be observed under a microscope. The results are shown on Figure 3.

3.4 Molecular analysis

3.4.1 Genomic DNA Extraction (Boiling Cell Method)

Extraction of DNA using the boiled-cell method with minor modification was used to detect the *E.coli* O157:H7 in the water sample provided by Chai *et al.* (2007). A single colony from the nutrient agar was transferred into the Luria Bertani (LB) broth and incubated for 24 hours at 37 °C. For each positive broth, 1.5 mL was centrifuged at 10000 rpm for five minutes to get the pellet. Then, the pellet was suspended by adding 500 µL of sterile distilled water and vortex vigorously to dissolve the pellet. Next, it was boiled for 10 minutes at 97°C and allowed to cool at -20°C for 5 minutes. The tube was centrifuged again at 10000 rpm for 10 minutes and the supernatant was used as DNA template for PCR assay.

3.4.2 Polymerase Chain Reaction (PCR)

For detection of *E.coli* O157:H7, PCR assay was carried out according to Kading et al (2011). A total of 25µl reaction mixtures containing 1x PCR buffer (Promega), 5 mM deoxynucleoside triphosphate (dNTP) mix (Thermo Scientific), 2.5 mM MgCl, 10 µL of each primer, 2 U of Taq polymerase and 5µL of DN A for E. coli were used to perform PCR amplification as shown in Table 3.1. The primers and PCR

conditions as outlined in Table 3.2 and Table 3.3 according to Meng *et al.* (1997) were utilized to amplify the stx1 gene in *E.coli* O157:H7.

Table 3.1 Master Mix component for PCR

Component	1x reaction (μL)
5x Green Go Taq®Flexi Buffer (5µ/µl)	5.0
25 mM Magnesium Chloride ($MgCl_2$)	2.5
10 mM Deoxyribonucleotide oligophosphates (dNTPs)	1.25
Forward Primer	1.0
Reverse Primer	1.0
DNA template	5.0
Sterile distilled water	8.85
Go Taq® Flexi DNA Polymerase (5µ/µl)	0.4
Total Volume	25.0

Table 3.2 Characteristic of primers to detect stx1 gene *E.coli* O157:H7

Primer	Nucleotide sequence	Target gene	Amplicons size (bp)
Slt1 (Forward Primer)	TGT AAC TGG AAA GGT GGA GTA TAC	*Stx*1 gene	210
Slt1 (Reverse Primer)	GCT ATT CTG AGT CAA AAA ATA AC		

Table 3.3 PCR condition for *E.coli* O157:H7

Step	Temperature	Time	
Initial denaturation	95.0	5 minutes	
Denaturation	94.0	30 seconds	30 cycles
Annealing	47.0	30 seconds	
Extension	72.0	90 seconds	
Final extension	72.0	5 minutes	

3.4.3 Agarose Gel Electrophoresis (AGE)

For 1% concentration gel will be used whereby 0.75 g of agarose powder in 50 mL of 1x TBE buffer. The mixture was microwaved at high temperature for 1 minutes and left to cool down. Pre-staining will be done on the gel throughout the whole project. Then, a 5 μL of PCR products were loaded onto the agar and undergo electrophoresis for 47 minutes at 90V. Ultra Violet (UV) transilluminator were used to view the PCR product after staining with ethidium bromide (EtBr). A 1kb basepair DNA ladder (Vivantis) was used to show the size of DNA fragments. The result for PCR products for detection of *stx* gene in *E.coli* O157:H7 with expected size 210 base pair from different restaurants on gel electrophoresis was shown in Figure 4.