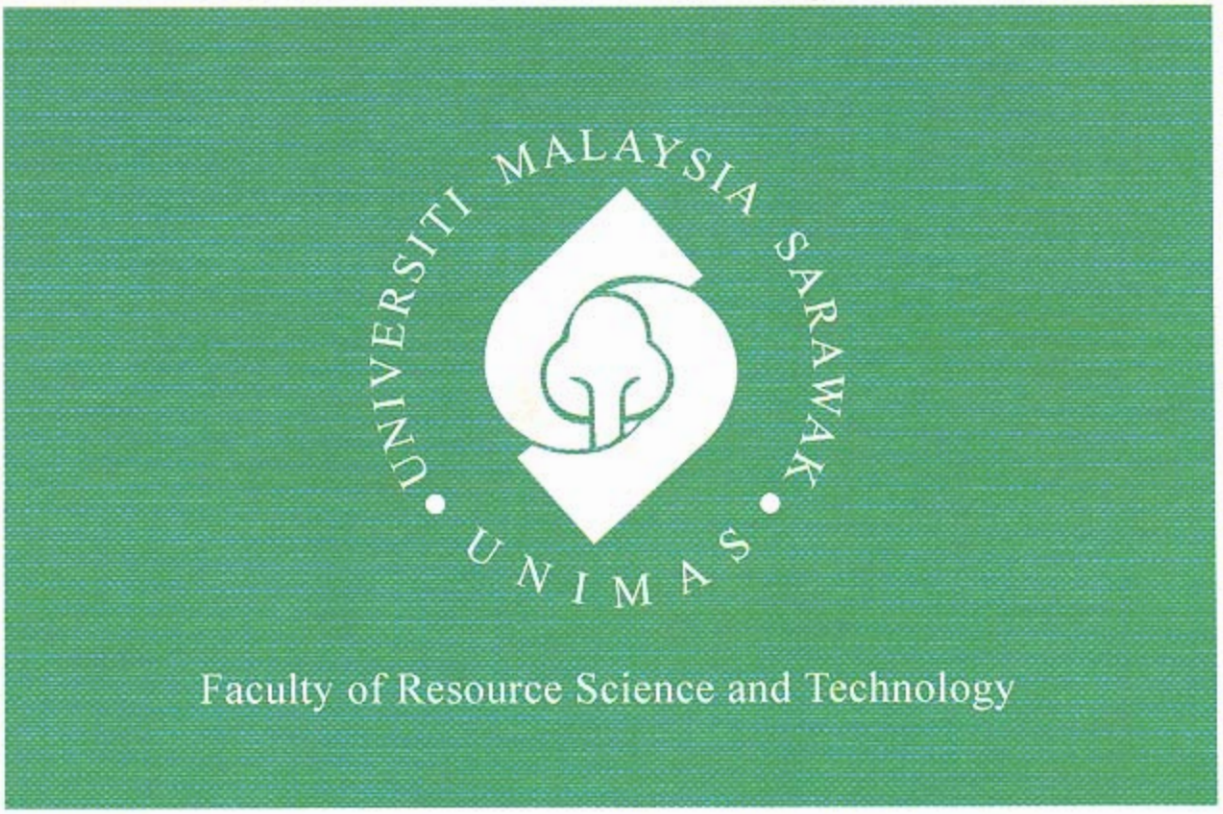

CHARACTERIZATION OF *Escherichia coli* ISOLATED FROM VILLAGE CHICKEN AND SOIL SAMPLES

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Bachelor of Science with Honours
(Resource Biotechnology)
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-Thank you all-

DECLARATION

I hereby declare that this Final Year Project Report is solely my original work which is based on this study. All citations and references mentioned in this final year project report have been acknowledged according to the respective guidelines.

Student's Signature

26 MAY 2011

Date

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LIST OF ABBREVIATIONS

°C	degree Celcius
EHEC	enterohemorrhagic *E.coli*
EMB	Eosin Methylene Blue
E. coli	*Escherichia coli*
kb	kilo base pairs
hrs	hours
MR-VP	methyl red and Voges-Proskauer
µl	microlitre
%	percentage
rpm	revolution per minute
*stx*1	Shiga-toxin gene 1
*stx*2	Shiga-toxin gene 2
STEC	Shiga toxin-producing *E.coli*
RAPD	Random Amplified Polymorphism DNA

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Characterization of *Escherichia coli* Isolated from Village Chicken and Soil Samples

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ABSTRACT

Escherichia coli (*E. coli*) is one of the known major causative agents causing food-borne diseases such as food poisoning and diarrhea. Various transmission agents such as poultry had been known to transmit diseases to human mainly through food consumption. This study was conducted to determine the genetic diversity, antibiotic susceptibility and the presence of Shiga-like toxin (SLT) genes in *E. coli* isolated from village chickens and soil samples. Samples of village chicken feces and soil were collected and transported to the lab and analyzed for the presence of *E. coli*. Samples were then plated on EMBA and colonies showing metallic green sheen were isolated and confirmed by biochemical test. Overall, 32 *E. coli* isolates were isolated from the samples and these isolates were analyzed by $(GTG)_5$ PCR, multiplex PCR and antibiotic susceptibility tests. The antibiotic susceptibility revealed that 100% of the isolates were resistant to erythromycin. *E. coli* isolates tested with chloramphenicol has the lowest level of resistance (6.3%). Through $(GTG)_5$ PCR analysis, it is shown that all *E. coli* isolates were genetically diverse. There were no Shiga-like toxin (SLT) genes detected in all 32 *E. coli* isolates. The multiple antibiotic resistance of *E. coli* isolated in this study indicates the potential health hazard associated with village chicken and its surrounding environment.

Keywords: *E. coli*, village chickens, antibiotic, Shiga toxin genes (*stx*1 and *stx*2), $(GTG)_5$ PCR

ABSTRAK

Escherichia coli (E. coli) merupakan salah satu agen penyebab utama penyakit berasaskan makanan seperti keracunan makanan dan diarrhea. Terdapat beberapa agen transmisi seperti ayam didapati boleh membawa penyakit kepada manusia melalui pemakanan. Kajian ini dijalankan untuk mengenalpasti kepelbagaian genetik, ketahanan antibiotik dan kehadiran gen toksin Shiga (SLT) di kalangan E. coli yang dipencil daripada sampel najis dan tanah. Sampel najis dan tanah dikumpul sebelum dibawa ke makmal untuk analisa kehadiran E. coli. Sampel telah diproses ke atas EMBA dan koloni berwarna hijau metalik telah dipencilkan dan dikenalpasti melalui ujian biokimia. 32 E. coli telah dianalisa dengan $(GTG)_5$ PCR, PCR multipleks dan ujian ketahanan antibiotik. Melalui ujian ketahanan antibiotik, 100% sampel menunjukkan kerintangan terhadap antibiotik eritromisin. E. coli yang telah diuji dengan kloramfenikol menunjukkan tahap ketahanan terendah (6.3%). PCR $(GTG)_5$ menunjukkan bahawa kesemua E. coli adalah berbeza dari segi genetik. Tiada gen toksin Shiga (SLT) dikenalpasti di kalangan kesemua 32 E. coli. Kesemua E. coli yang telah dipencil menunjukkan ketahanan antibiotik berganda dan ini berpotensi untuk menyebabkan masalah kesihatan berkaitan dengan ayam kampung dan persekitarannya.

Kata kunci: E. coli, ayam kampung, antibiotik, gen toxin Shiga (stx1 dan stx2), PCR $(GTG)_5$

1.0 INTRODUCTION

Escherichia coli are enteric bacteria that can be found in the human intestinal tracts where they survive as natural inhabitants and are harmless towards human body system. *E. coli* are common examples of coliform bacteria usually present in environments and in warm-blooded organisms such as humans (Daniels & Easterly, 2008). Nevertheless, some *E. coli* strains such as *E. coli* (EHEC) serotype O157:H7 are pathogenic and have great potential in causing health problems to humans. Food poisoning is among the highly concerned health problem related with *E. coli*. An increasing numbers of cases regarding food-borne diseases caused by *E. coli* had now become a global concern. Hemorrhagic colitis and hemolytic uremia syndrome (HUS) which is also known as bloody diarrhea are among the disease reported (Sahilah *et al.*, 2010b).

Various mode of transmission of pathogenic *E. coli* such as through consumption of food and water and also by human-to-human transmission leads to rapid disease spreading (Radu *et al.*, 1998). Unintentional food consumption contaminated with *E. coli* will soon develop the symptoms of diarrhea. Lacking of personal hygiene concern also contribute to infection caused from *E. coli*. Poultry based food especially retail chicken is most likely to be reservoirs for *E. coli* serotype O157:H7. Although there is not yet any major outbreak reported in Malaysia, *E. coli* O157:H7 had been isolated from beef samples collected from retail market (Radu *et al.*, 1998). Diverse bacteria with high genetic diversity including *E. coli* are present in soil as well as in water sources.

Various treatments had been applied to cure health problems following food-borne diseases. One of the treatments is by giving out antibiotics to patients. However, after a prolonged usage of antibiotics, it is possible that a novel bacterial strain with the ability to resist antibiotic activity will develop. In addition, multiple antibiotic resistant bacteria can also develop thus making the infection harder to be treated. Horizontal gene transfer among *E. coli* and other pathogenic bacteria such as *Vibrio cholera* and *Salmonella typhi* can also lead to development of novel bacterial strains. Antibiotics are also incorporated into feeds of commercial chicken in order to treat diseases (Miles *et al.*, 2006). Recent advancement in microbial screening leads to the efficiency of various bacterial strains typing as well as the detection of toxin genes in bacteria isolates.

The objectives of this study are:

1. To isolate and identify *E. coli* from village chickens and soil.
2. To determine the antibiotic resistance among the *E. coli* isolates.
3. To analyze the genetic diversity of the *E. coli* isolates among different samples from different locations.
4. To detect the presence of Shiga toxin (*stx*1 and *stx*2) genes in the *E. coli* isolates.

2.0 LITERATURE REVIEW

2.1 Village chickens and environment

Village chickens or *Gallus domesticus* are free range chickens that are not properly raised for which they usually lived in a poorly conditioned environment. These village chickens are also not well-fed compared to chickens raised for commercial purposes. Most of the time, they consume foods around their habitats. Thus, this poor condition usually causes the infection of *E. coli* among poultry. Presence of *E. coli* in village chickens is expected as poultry are common reservoirs of the bacteria. The presence of *E. coli* in village environment is also a common occurrence and these *E. coli* could be pathogenic. Health issues especially those related with food-borne disease will occur once these pathogenic *E. coli* had been infected to humans or animals. *E. coli* isolates with similar diversity can be detected by various molecular methods such as $(GTG)_5$ PCR. These similarities can be related with genetic cross contamination leading to genetic transmission during transportation of village chicken to new locations. Other modes of transmission such as via conjugation or transformation are also the main causative mechanism of occurrence of *E. coli* in both village chicken and the environment. By these mechanisms, village chickens carrying pathogenic strain of *E. coli* will infect other village chicken in the new location.

2.2 *Escherichia coli*

Escherichia coli, commonly abbreviated as *E. coli* was first discovered by Thoedor Escherich who was a German pediatrician and bacteriologist. *E. coli* is a gram negative bacteria and a rod-shaped bacterial species that belongs in the *Enterobacteriaceae* family. *E. coli* is usually found in the human intestinal tract and is pathogenic towards human health once they are infected. Nevertheless, there are harmless *E. coli* strains that are able to survive in the intestines by benefiting from its host. *E. coli* contamination in food is among the main source that contributes to health problem. Food-borne disease had occurred numerous times due to this problem. Pathogenic *E. coli* strain causing food-borne diseases usually found in fecal (Jay, 1992). Cases regarding retail poultry products contaminated by avian fecal *E. coli* are usually serious (Johnson *et al.*, 2003). These cases arise mostly due to the lack of hygiene concern during food preparation and handling. In addition, consumptions of undercooked beef, raw milk as well as unpasteurized juices are among the source of food contamination due to the presence of *E. coli* in those defect products. *E. coli* infection is rather rapid due to the short incubation period thus patient shows rapid signs of sickness.

2.3 *E. coli* strains

E. coli with the potential to cause food-borne disease can be classified into five different groups; enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC) or facultative enteropathogenic (FEEC) (Jay, 1992).

EPEC *E. coli* could cause diarrhea although no enterotoxins are produced whereas EIEC produces enterotoxins. The mechanism of association of EPEC with intestinal mucosa involves attachment-effacement that eventually leads to interruption to the epithelial cell membrane (Jay, 1992). In contrast with the previous strains, both ETEC and EHEC produced enterotoxins. Heat-labile (LT) comprises of subunits A and B (LTA and LTB) and heat-stable (STa or ST-I and STb or ST-II) enterotoxins are produced by ETEC. This strain also produces colonizing factors antigens characterized by fimbriae (or pili) in order to aid in the adherence of cells to epithelial cells (Jay, 1992). ETEC frequently carry plasmids with antibiotic resistance, enterotoxins and also binding antigens. EHEC is the most common causative agent that can cause diarrhea in human as these *E. coli* strains inhabit the human intestine. *E. coli* O157:H7 is an example of EHEC *E. coli* strain producing SLT-I and SLT-II. To facilitate the serotyping of *E. coli* strains such as *E. coli* O157:H7, three antigens are utilized; O antigen which is a heat-stable somatic antigen, K antigen which is a heat-labile somatic antigen and H antigens which is a heat-labile flagellar antigen.

2.4 SLT-I and SLT-II in *E. coli*

One of the known pathogenic *E. coli* strains is the serotype O157:H7 which carry Shiga toxins encoded by *stx*1 and *stx*2 genes (Ferens *et al.*, 2006). The presence of *stx*1 and *stx*2 genes were detected in *E. coli* isolated from avian sample (Parreira & Gyles, 2002). Despite that, from all the isolates obtained in that study, only 52% of the isolates were detected with both Shiga toxin genes. Whereas, only one isolates was detected with *stx*2 gene meanwhile *stx*1 gene was detected in the remaining isolates (Parreira & Gyles, 2002). A considerable frequency of *E. coli* O157:H7 strains processing virulence traits were shown to be present in retail beef marketed in Malaysia (Radu *et al.*, 1998). Shigella genus belongs to *Enterobacteriaceae* with four common species such as *S. dysenteriae, S. flexneri, S. bodyii and S. sonnei* (Jay, 1992). *Shigella dysenteriae* is pathogenic due to their ability to produce Shiga toxins. *Shigella dysenteriae* can either belong to shigatoxigenic group of *E. coli* (STEC) or enterohemorrhagic *E. coli* (EHEC) serotype O157:H7. STEC is a vero toxin-producing *E. coli* (VTEC) O157:H7 strains that usually cause hemorrhagic colitis (HC) (Al-Darahi *et al.*, 2008). The HC causes gastrointestinal disease such as bloody diarrhea and it will lead to haemolytic uremic syndrome (HUS). The verocytotoxin or verotoxin are also equivalently known as Shiga-like toxin (Meng *et al.*, 1997) and are active on Vero cells. The site of action of Shiga toxin genes is usually at the lining of the blood vessels of digestive tract. All SLTs are cytotoxic to Vero cells (Jay, 1992). Anti-Shiga antisera are used to neutralize SLT-I and Shiga toxin. Some SLT-II variants are toxic to Vero cells and they are neutralized by antisera against SLT-II and not by anti-Shiga toxin (Jay, 1992). PCR assays are highly sensitive and specific in microbial pathogens detection thus it is one of the few methods developed in order to detect *E. coli* O157:H7 (Rolfs *et al.*, 1992; Meng *et al.*, 1994).

2.5 Antibiotic resistance

For the purpose of this study, disk diffusion method was utilized according to National Committee for Clinical Laboratory Standards (NCCLS) (1997). *E. coli* ATCC 25922 is usually used as reference strains in most studies involving antimicrobial susceptibility test.

Antibiotic resistance is referred to as the ability of bacteria to withstand antibiotic action. Antibiotic resistance among bacterial strains mainly in *E. coli* isolates derived from poultry product such as retail chickens is increasing significantly. More diseases which are potentially carried by chickens are not being treated well due to the widespread antibiotic usage. Frequent usage or prolonged exposure of antibiotic against bacterial infection as treatment causes the bacteria strains to be resistant against antibiotic. Application of various antibiotics to reared animals such as chickens often lead to multi-resistant *E. coli* towards antibiotic. Bacteria such as *E. coli* which are resistant towards antibiotic usually inhabit the fecal flora of animals which usually contains high proportion of resistant bacteria (Miles *et al.*, 2006).

Regardless of the fact that bacteria can obtain resistance genes upon the exposure to antibiotic usage, some bacteria are known to have natural resistance properties in their genetic makeup (Davison *et al.*, 2000). Resistance genes are found in the chromosomes, transposons and plasmids which can be transmitted by various mechanisms (Davison *et al.*, 2000). The mechanism of transmission of antibiotic-resistant gene from animals to human is often through the infection of *E. coli* in the human intestinal tract. Spreading of plasmids carrying antibiotic-resistant genes can easily occur once the human intestinal tract had been infected.

Resistance gene transfer can occur both vertically and horizontally or via mutations. The gene transfer among bacteria of different genera and families occur vertically meanwhile horizontal gene transfer occur between different bacterial species. Resistance towards antibiotics is found in soil in which it is acquired as a result of environmental exposure. Bacteria in the soil will then create a reservoir for resistance factors (Miles *et al.*, 2006).

It was found that *E. coli* isolated from retail chicken products are resistant to nalidixic acid and thus they are potentially become the transmission vehicle of *E. coli* into humans (Johnson *et al.*, 2003). Quinolone resistance was reported as a result from chromosomal mutations in DNA gyrase and alteration of DNA topoisomerases (Miles *et al.*, 2006). These alterations will lead in the reduction of protein membrane permeability of antimicrobial agents thus resulted in difficulty to treat infections (Khan *et al.*, 2005). It was reported that enteric *E. coli* isolated from calves, multiple antibiotic resistance has developed following the exposure to feeds such as milks that were incorporated with antibiotics (Berge *et al.*, 2005).

2.6 Epidemiology

Pathogenic bacteria such as *E. coli* that are able to cause disease such as bloody diarrhea (HUS) can be transmitted from poultry to human by various transmission modes. Nevertheless, consumption of contaminated food based from poultry products are among the major contributor leading to transmission of diseases caused by *E. coli* bacteria. Poultry are known to be common reservoirs for pathogenic *E. coli* bacterial strains. The chances of Shiga toxin genes existence in village chickens including the environment where they lives is suspected to be significant as these species have not been raised properly. Nearby stream can carry feces of village chickens infected with *E. coli.* Cattles are also one of the major reservoirs of pathogenic *E. coli* O157:H7 that had been linked to disease outbreaks resulted from consumption of bovine origins products. Beef meat with prevalence of STEC from local markets may serve as transmission vehicle to human (Sahilah *et al.*, 2010a).

Study of interaction between organisms and also with their host in the same or different population can aid in the better understanding of resistance variation between isolates. The various resistance results can be obtained after antibiotic susceptibility test had been carried out. In addition, the mode of resistance gene transfer can also be further studied in order to identify whether horizontal or vertical gene transfer are involved (Miles *et al.*, 2006). In addition, conducting a serotyping approach to *E. coli* strains can be useful in better understanding of their virulence properties (Jay, 1992).

2.7 Typing of *E. coli*

The significance of *E. coli* isolates typing is important for better understanding especially the pathogenic *E. coli* O157:H7 strain during outbreaks. RAPD-PCR utilizes primers of arbitrary GC-rich decamers (10-mers) (Radu *et al.*, 2001). RAPD-PCR method is more sensitive and cost effective for typing and differentiating *E. coli* isolates (Salehi *et al.*, 2008). The molecular characterization of the Shiga toxin-producing *E. coli* (STEC) is determined by means of RAPD-PCR (Kim *et al.*, 2005). Various PCR-based profiling techniques have been applied in many researches to observe the genetic diversity and also epidemiology relationships of *E. coli*. Among them are RAPD-PCR, amplified restriction fragment length polymorphism (AFLP), plasmid profiling, pulsed-field gel electrophoresis (PFGE), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), and multiplex-PCR (Sahilah *et al.*, 2010b; Radu *et al.*, 2001). PCR require primers for the binding of polymerase to the nucleic acid template as well as to direct the movement of polymerase from 5' to 3' directions for DNA amplification. A multiplex PCR was applied in order to detect *E. coli* O157:H7 from tenderloin beef and chicken meat burger (Radu *et al.*, 2001). The confirmation of *E. coli* O157 serotype H7 was made by combining a pair of primer for SLT-I and SLT-II genes with another pair of primer for H7 flagellar gene. PFGE is then carried out for the characterization of the *E. coli* isolates. PFGE analyzes the whole length of chromosome of amplified DNA, whereas RAPD only analyzes random parts of the chromosome. However, both methods had shown evenly good results in strain differentiation. Identification of *E. coli* O157:H7 was made using a multiplex PCR assay developed by utilizing primers that amplify a DNA fragment upstream of *E. coli* O157:H7 *eae*A gene and SLT genes (Meng *et al.*, 1997). This assay can be applied to detect *E. coli* O157:H7 strains especially in food.

2.7.1 $(GTG)_5$ PCR application

The application of $(GTG)_5$ PCR technique is beneficial especially in molecular work for DNA fingerprinting where the genetic diversity of different isolates is determined. Moreover, the origins of bacteria species and their relatedness can be determined through this method. Application of oligonucleotide primer $(GTG)_5$ is necessary for the purpose of obtaining amplified PCR product (Matsheka *et al.*, 2005) From AGE performed for $(GTG)_5$ PCR, multiple banding patterns are observed and these bands represent the profiles of the diverse isolates. This technique implements the phenotypic characteristics of isolates and the results can be analyzed through the plotting of dendogram. From the dendogram, the evaluation can be done by determining the genetic diversity distance represented by clusters. Repetitive extragenic palindromic-PCR coupled with $(GTG)_5$ PCR was able to determine the source of fecal pollution by *E. coli* (Mohapatra *et al.*, 2008). $(GTG)_5$ PCR technique is a rapid and excellent genotypic tool for enterococci and lactobacilli typing (De Vuyst *et al.*, 2008). In molecular typing of fecal and environmental *E. coli* isolates, it was reported that $(GTG)_5$ genomic PCR is the most appropriate method to be applied (Mohapatra *et al.*, 2007). Moreover, this technique is a cost-effective and easy to perform method for epidemiology studies (Matsheka *et al.*, 2005).

3.0 MATERIALS AND METHOD

3.1 Sample collection

Samples of village chicken feces and soil were collected from various villages such as Tasik Biru residential area, Kpg. Sagah Singgai, and Kpg. Sungai Moyan within four months period (October 2010 – January 2011). Four samples comprised of at least two feces and two soil samples were collected from each village. All samples were labelled and stored in sterile media bottles and placed inside a polystyrene box containing ice packs. Aseptic technique was applied during sampling. The samples were transported to the Microbiology Laboratory, UNIMAS for sample processing and *E. coli* isolation.

3.2 Preparation of Eosin Methylene Blue Agar (EMBA)

Before all the samples were processed, EMBA was prepared. EMBA plates were used for the isolation of *E. coli* from the samples. In order to prepare 1000 ml of EMBA, 36 g of EMB agar were weighed, dissolved in 1000 ml of distilled water and autoclaved at 121 °C, for 15 minutes. Nonetheless, only required amount of EMBA powder for the samples plating were prepared, sterilized and poured into petri dishes. The EMBA plates which were not used immediately were stored at 4 °C inside a fridge until further usage.

3.3 Sample processing and isolation of *E. coli*

The samples were immediately processed upon arrival in laboratory. Prior to serial dilution procedure, 0.85% saline solution was prepared. In order to prepare 250 ml of 0.85% saline solution, 2.125 g of NaCl was required. Serial dilution was done at dilution factors of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Nine millimetres of each saline solutions were pipetted into test tubes and sterilized by autoclaving at 121 °C for 15 minutes prior of usage. One gram of each sample was weighed and mixed into the test tubes containing sterilized 0.85% saline solution. Then, 100 µl of the serial dilutions ranging only from dilution factors of 10^{-2}, 10^{-3} and 10^{-4} containing samples were spread plated onto EMBA. All plates were incubated at 37 °C overnight. After overnight incubation, each plate was observed for the growth of *E. coli* and colonies grown on the EMB agar were counted and recorded. EMBA plates containing 30-300 colonies of *E. coli* were selected and about 5-10 colonies were picked for further *E. coli* purification step.

3.4 Stock Culture

Before making stock culture, 3 – 5 single colonies of presumptive *E. coli* grown on previously spread plated EMBA were picked and re-streaked onto new EMB agars to produce pure culture of *E. coli*. Once a single colony is present on the EMBA, it was inoculated on agar slant of Luria-Bertani agar (LBA) slant for stock culture. The inoculated LBA slants were incubated at 37 °C to allow the growth of purified *E. coli* isolates. After incubation, the agar slants containing bacterial growth were kept in a fridge as stock culture for further usage.

3.5 Confirmation tests of *E. coli* isolates

3.5.1 Gram staining

A gram staining procedure was carried out to distinguish between two major groups of bacteria as well as to confirm the growth of *E. coli* isolates. Firstly, all *E. coli* isolates from each sample were inoculated from stock culture using a sterilized inoculating loop and streaked onto LBA plate to obtain a single colony. After an overnight incubation, a single colony from all samples was picked and smeared onto a microscope slide in a circular motion. The bacteria smear was then heat-fixed to the slide and the slide was placed on a staining rack. The staining steps are as shown in Figure 1. First staining was done with few drops of crystal violet for 1 minute. After 1 min, the crystal violet was rinsed off from the microscopic slide with distilled water. This was followed with staining the slide with few drops of Gram's iodine for 1 minute and later was rinsed off with distilled water. Next, the slide was briefly stained using acetone to decolorized the bacterial smear and for 30 seconds. The acetone was then completely rinsed off from the slide. The final staining was done using few drops of safranin counterstain for 2 minutes. After 2 minutes, the safranin counterstain was completely rinsed off with distilled water. After Gram's staining is done, all slides were observed under a microscope at magnification of 100X. Therefore, *E. coli* can be identified based on the colour of the smear on the slide after Gram's staining. The shape of the bacteria on the slides was also identified. As *E. coli* is a gram-negative bacterium, the bacterial smear should appear pinkish with the shape of bacilli.

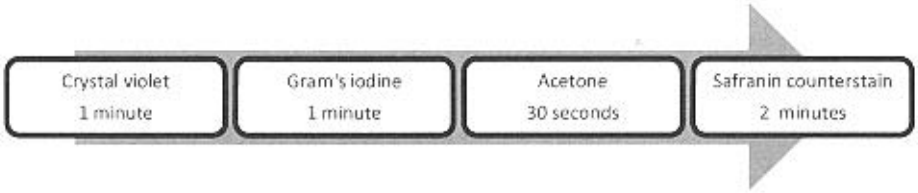

Figure 1: Gram's staining steps

3.5.2 Biochemical tests

A series of biochemical tests were carried out to confirm the *E. coli* isolates. Among the biochemical tests carried out were methyl red-Voges-Proskauer (MR-VP), indole, motility, hydrogen sulphate (H_2S) and citrate tests.

MR-VP medium was prepared in test tubes and sterilized prior of usage in the MR-VP tests. For MR-VP tests, single colony of presumptive *E. coli* were inoculated from LBA slants stock culture into MR-VP medium. The inoculated MR-VP medium was incubated at 37 °C for 48 hrs in a shaker incubator. After 48 hrs of incubation, the growth of *E. coli* in MR-VP broth was observed. Five drops of methyl red solution were dropped into the inoculated methyl red medium and was left for 1 hour. Colour changes of the MR medium from yellow to cherry red indicate the positive result of methyl red for *E. coli*. For Voges-Proskauer test, six drops of Barritt's-A (α-naphtol) reagent were dropped into the inoculated VP medium. This was followed by dropping two drops of Barritt's B (potassium hydroxide) reagent into the test tubes. No colour change in VP medium indicates the positive result of *E. coli*.