Faculty of Resource Science and Technology

ANTIBIOTICS RESISTANT AND GENETIC DIVERSITY OF *Escherichia coli* ISOLATED FROM AQUACULTURE ENVIRONMENTS

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DECLARATION

I hereby declare that no portion of this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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LISTS OF ABBREVIATIONS

^{0}C	Degree celcius
%	Percentage
DNA	Deoxyribonucleic acid
ng	Nanogram
g/L	Gram/litre
µl	Microlitre
mm	Millimeter
min	Minutes
g	Gram
ml	Milimitre
rpm	Revolution per time

LIST OF TABLES AND FIGURES

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ABSTRACT

The use of antibiotics in the aquaculture environments poses a risk that resistance to antibiotics will develop in the environmental organisms, in the endogenous bacterial population of farmed species, and in their pathogens. Antibiotic resistance bacteria constitute direct or indirect threat to farmers and the public through potential transfer of resistance to human and animal pathogens. This study was conducted to obtain a preliminary indication of the extent of antibiotic resistance in the aquaculture environments in Sarawak. *Escherichia coli* were isolated from the fish, water, and the sediment of the aquaculture environments. Antibiotic susceptibility test was conducted to determine the antibiotic resistance patterns of the isolates. DNA profiling of *E. coli* was conducted to see the diversity of the isolates in the aquaculture environment through RAPD-PCR analysis. Nineteen isolates of *E. coli* were isolated from the fish, water and sediment. All the isolates show resistant to at least one of the eight antibiotics tested. The highest level of resistance was observed against erythromycin (94.74%) and the lowest was against nalidixic acid (5.26%). The RAPD analysis with primers Gen-1-50-09 and Gen1-50-10 grouped the *E. coli* isolates in 4 and 3 main groups, respectively. The results of this study suggested that *E. coli* isolates were genetically diverse and the fish, sediment and water are potential reservoir for antibiotic resistance of *E. coli*.

Keywords: *Escherichia coli, a*ntibiotic, RAPD-PCR

ABSTRAK

Penggunaan antibiotik dalam bidang akuakultur menimbulkan risiko ketahanan antibiotik pada organisma, populasi bakteria dalam spesis yang dikultur dan juga terhadap pathogen spesis yang dikultur tersebut. Bakteria yang tahan terhadap antibiotik turut membahayakan penternak dan warga umum secara langsung ataupun tidak langsung melalui pemindahan gen daya tahan antara manusia dan patogen. Kajian ini telah dijalankan untuk mengenalpasti petunjuk awal sejauh mana daya tahan terhadap antibiotik dalam akuakultur dan persekitarannya. Escherichia coli telah dipencilkan daripada sampel ikan, air dan mendakan. Ujian kepekaan terhadap antibiotik dijalankan untuk menentukan corak ketahanan E. coli dalam persekitaran akuakultur melalui analisis RAPD-PCR. Sembilan belas E. coli telah dipencilkan daripada ikan, air dan mendakan. Kesemua E. coli yang telah dipencilkan menunjukkan kerintangan terhadap sekurang-kurangnya 1 antibiotik daripada 8 antibiotik yang telah digunakan. Tahap ketahanan yang paling tinggi adalah terhadap eritromisin(94.74%) manakala yang paling rendah adalah terhadap asid nalidisik (5.26%). Analisis RAPD menggunakan primer Gen1-50-09 dan Gen1-50-10 masing-masing menggumpulkan E. coli yang telah dipencilkan kepada 4 dan 3 kumpulan utama. Keputusan daripada kajian ini mencadangkan E. coli yang telah dipencilkan secara genetiknya adalah pelbagai dan ikan, air dan mendakan adalah berpotensi untuk bertindak sebagai punca kepada E. coli tahan antibiotik.

Kata kunci: *Escherichia coli,* antibiotik, RAPD-PCR

1.0 INTRODUCTION

Antibiotics are generally used to prevent or treat e bacterial diseases. The incorrect use of those antimicrobial agents for therapeutical purposes in veterinary science may lead to the development of antibiotic resistance bacteria. Antibiotics resistance genes are coded by chromosomal DNA and it can also be carried by the extrachromosomal DNA, which is the plasmid. The presence of antimicrobial agents at low concentration through leaching or continued usage may lead to the development of drug-resistant strains and multiple antibiotic resistance (MAR) in bacteria, which may result to resistance transfer to pathogenic bacteria and reduced efficacy of antibiotic treatment for human and animal diseases (Tendencia *et al*., 2000). DNA coding for antibiotic resistance may be conjugally transferred between similar bacteria. In many cases the genetic code for antibiotic resistance is placed on so-called R-plasmids (Hirsch *et al*., 1999).

There are several studies that have been conducted to investigate any possibility of the used of antimicrobial agent to the organisms. Most of the studies are focused on fish and its environment. Spanggaard *et al.* (1993) reported resistance to oxytetracycline among bacteria from freshwater fish farms in Denmark. As has been reported by Nygaard *et al*. (1992) exposure to oxytetracycline and oxolinic acid initiates resistance to other drugs. McPhearson *et al*. (1991) observed that individual and multiple antibiotic resistances were associated with antimicrobial use. Antibacterial-resistant bacteria in surficial sediments near salmon net cage farms were isolated by Herwig *et al*. (1997).

The use of antibiotics in aquaculture poses specific dosage problems, when aquatic species are farmed at high densities, individual dosing is impractical and the alternatives are either addition to feeds or to the water. In both cases this results in the introduction of antibiotics at varying concentrations in the aquatic environment in the proximity of farms and

this situation is likely to trigger the selection and dissemination of resistance to these antibiotics in the autochthonous bacterial population of aquaculture sites.

RAPD-PCR analysis with an arbitrary primer sequences allowed researcher to study the differences among organisms. According to Ruiz *et al*. (2000) banding patterns from an individual or population can then be used as a fingerprint that may distinguish it from other genetic types.

The objectives of this project are:

1) To isolate *E. coli* from aquaculture environment.
2) To determine the antibiotics resistance patterns among the *E. coli*.
3) To conduct DNA profiling of the *E. coli* isolated from aquaculture environment.

2.0 LITERATURE REVIEW

2.1 Aquaculture Industries

According to Kinsey (2006), aquaculture is defined as the systematic cultivation of the natural produce of aquatic habitats. Aquafarming involves the cultivating aquatic populations under controlled conditions. Landau (1993) stated that aquaculture is the raising and harvesting of fresh and saltwater plants and animals. The most economically important form of aquaculture is fish farming, an industry that accounts for an ever increasing share of world fisheries production. Formerly a business for small farms, it is now also pursued by large agribusinesses, and by the early 2000s it had become almost as significant a source of fish as the as wild fisheries (Landau, 1993).

Common products of aquaculture are catfish, tilapia, trout, crawfish, oysters, shrimp, and salmon, and tropical fish for aquariums. Some are raised in huge freshwater tanks or ponds, meanwhile others require the running water of rivers or streams. Saltwater species are often raised in saltwater ponds, in enclosed bays, or in pens placed in coastal or deeper sea waters. Aquaculture environment has develop rapidly especially in Malaysia. According to Ng (2009), the fisheries sector in Malaysia contributed 1.42 million tons of seafood valued at RM 5.3 billion (USD 1 = RM 3.4) in 2007. The presence of vast bodies of inland freshwaters and the long coastline in Peninsular Malaysia, Sabah and Sarawak also augments well for future aquaculture development, (Ng, 2009). Traditionally, in commercial aqua feeds, fish meal and fish oil are used as the major source of dietary protein and lipid, respectively.

2.2 *Escherichia coli*

The bacterial species *E. coli* is one of the most common inhabitants of the human intestinal tract and is probably the most familiar organism in microbiology. Ishii *et al.* (2006) reported that *E. coli* originally thought to be present only the intestinal tracts of warm blooded animals including humans. Recently, *E. coli* has been found in tropical and subtropical soils but under controlled laboratory condition, (Ishii *et al.*, 2006). Its presence in water or food is an indication of fecal contamination and usually not pathogenic. Hansen *et al.* (2008) has reported that the presence of *E. coli* is currently used as an indicator of recent fecal contamination in recreational waters. Molinari *et al*. (2003) has also reported that bacteria in the genera *Aeromonas, Burkholderia, Chromobacterium, Citrobacter, Escherichia, Flavimonae, and Plesiomonas* are present in farm-raised tilapia. Several studies have been conducted on the presence of the fecal coliform in farm reared fish because of concern about the health of fish consumers, (Hansen *et al.*, 2008).

According to Ksoll *et al.,* (2007) an ever-increasing number of studies completed during the past 40 years have provided evidence indicating that fecal coliforms and *E. coli* can persist in secondary, nonhost habitats and prolonged survival of fecal coliforms and *E. coli* in freshwater has been studied for several decades. In recent years, other studies indicate that *E. coli* can survive in sediments and soils over extended periods of time and from several Lake Michigan beaches was shown to harbor not only high densities of *E. coli* and enterococci but also potential human pathogens such as *Salmonella* and *Campylobacter* spp, (Ksoll *et al.*, 2007).

Eventhough it is not pathogenic, it can be a cause of urinary tract infections, and certain strains produce enterotoxins that cause traveler's diarrhea and occasionally cause very serious foodborne disease. Based on the previous reported by Zurek *et al*. (2004), outbreaks of the food-borne illness caused by *E. coli* O157:H7 have been reported throughout the

northern hemisphere, most frequently in the United States, Canada, Japan, and the United Kingdom. *E. coli* O157:H7 strains commonly carry verotoxins which encoded by the *stx1* and *stx2* genes and factors for the attachment to the host mucosa, including intimin, encoded by the *eaeA* gene (Zurek *et al.*, 2004).

Paton *et al*. (1998) reported that Shiga toxin-producing *E. coli* (STEC) strains have emerged as an important cause of serious human gastrointestinal disease, which may result in life-threatening complications such as hemolytic-uremic syndrome. Food-borne outbreaks of STEC disease appear to be increasing and, when mass-produced and mass-distributed foods are concerned, can involve large numbers of people, (Paton *et al*., 1998). Hauvelink *et al.* (2000) has also reported that Shiga toxin-producing *E. coli* (STEC) are an important cause of haemorrhagic colitis and the diarrhoea-associated form of the haemolytic uraemic syndrome. Of the numerous serotypes of *E. coli* that have been shown to produce Shiga toxin (Stx), *E. coli* O157:H7 and *E. coli* O157: NM (non-motile) are most frequently implicated in human diseases (Hauvelink *et al*., 2000).

E. coli also plays a vital role in genetic engineering technologies and industrial microbiology nowadays. Production of recombinant protein was successful by using plasmid, restriction enzyme and *E. coli*. Recently, production of human insulin was one of the useful applications of recombinant DNA technology by the manipulation of *E. coli*. According to Ingram *et al.* (1987) cloning of the gene coding for pyruvate decarboxylase from *Zymonas mobilis*, its sequence and its expression are high in *E. coli* and expression of *Z. mobilis* alcohol dehydrogenase II gene in *E. coli* has also been completed. *E. coli* is capable of actively metabolizing a wide variety of substrates, including hexoses, pentoses, lactoses. Previous study conducted by Ingram *et al.* (1987) has proved that replacement of the native enzymes for the production of fermentation products in *E. coli* with those from *Z. mobilis*

without a decrease in growth illustrates that the particular fermentation product produced is relatively innocuous to this organisms.

2.3 Antibiotics resistance

Based on the Martinez (2002) study, antibiotics are probably the most successful family of drugs so far developed for improving human health. To treat bacteriological infections, it is common practice to employ antibiotics such as oxytetracycline, enrofloxacin, sarafloxacin, and florfenicol (Aja *et al.,* 2002). According to Schwartz (2003), emergence of bacteria resistant to antibiotics is common in areas where antibiotics are used, but occurrence of antibiotic-resistant bacteria is also increasing in aquatic environments. Antibiotic substances may strongly affect quantitative and qualitative composition of *bacteriocenoses* in water ecosystems and may also play a substantial role in food competition systems (Barja *et al*., 1989; Lemos *et al*., 1991; Sabry *et al.,* 1997).

There is a perception that the usage of antibiotic to the aquaculture environment will affect the human the farmers. The concern is the potential of human pathogens, in interacting with the aquaculture environment, to acquire new resistance traits through genetic exchange with resistant subpopulations of the autochthonous microbiota. The extent of antibiotic use is indicative of the selection pressure exerted on bacteria (Schwartz *et al.,* 2003) many have developed various very efficient mechanisms to render ineffective the antibiotics used against them (Mudryk, 2004). In some regions, farmers of aquatic species would be unable to maintain a viable enterprise without the use of antibiotics.

There are many ways for the antibiotic susceptibility can be conducted. According to He *et al.* (2006) the disk diffusion method can offer a category result based on a zone size, meanwhile broth and agar dilution method can provide a minimum inhibitory concentration.

But those methods are time consuming, labour intensive and costly. Several automated systems have been used in clinical microbiology laboratories, such as MicroScan-WalkAway and the Vitek system (Burns *et al*., 2001; Dillard *et al*., 1996; Rittenhouse *et al*., 1996).

2.4 Rapid Amplified Polymorphism DNA (RAPD) -PCR Analysis

RAPD-PCR analysis is a molecular technique that used DNA template which is then selectively amplified thus producing large amount of concentrated DNAs or the PCR products, (Ruiz *et al*. 2000). Agarose gel electrophoresis will separate the PCR products into bands of different molecular weight. In recent years, RAPD has been used to characterize and trace the phylogeny of diverse plant and animal species. According to Smith *et al*., (2002) the genetic diversity of 33 Nigerian *Helicobacter pylori* isolates were studied using RAPD-PCR and Southern blot analysis of *ureA* or *ureCD* gene probes. In Africa, Kidd *et al*., (2001) reported on the use of RAPD and REP-PCR to assess the association between clonal grouping, disease and virulence fingerprints of 76 South African *H. pylori cag*A positive strains. Analysis of *E. coli* O157:H7 isolates from beef by using RAPD-PCR were previously conducted by Radu *et al*., (2000).

According to Hejazi *et al. (1997)* the polymerase chain reaction (PCR) has been demonstrated as a useful method for typing many micro-organisms and the PCR with random amplified polymorphic DNA (RAPD) analysis has been used successfully for typing different organisms, including some gram-negative bacteria. Previous study conducted by Quesada *et al.* (1995) was using RAPD-PCR analysis for the taxonomic identification of wine yeast strains such as *Saccharomyces, Candida, Pichia, Torulaspora, Hansenula,* and *Rhodotorula.* Application of RAPD-PCR also has been reported by Deak *et al.* (2000) in the molecular characterization of *Yarrowia lipolytica* and *Candida zeylanoides* isolated from poultry.

2.5 Agarose Gel Electrophoresis (AGE) analysis

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Agarose gel electrophoresis is routinely used for the preparation and analysis of DNA. It is a procedure that separates molecules on the basis of their rate of movement through a gel under the influence of an electrical field. Because nucleic acid molecules carry negative charges on their phosphate groups, they all travel toward the positive electrode in an electric field and as they move, the thicket of polymer fibers impedes longer molecules more than it does shorten one, separating them by length (Campbell *et al.* 2005). According to Campbell *et al.,* (2005), how far a DNA molecule travels while the current is on is inversely proportional to its length.

According to Brody *et al.* (2004) DNA electrophoresis has been a dominant technique in molecular biology for 30 years and the foundation for this common technique is based on a few simple electrochemical principles. Electrophoretic DNA separation from existing protein and RNA techniques developed DNA electrophoretic and conductive media remained largely unchanged, with Tris as the primary cation, (Brody *et al.,* 2004). DNA electrophoresis relies simply upon the negative charge of the phosphate backbone and the ability to distribute a voltage gradient in a sieving matrix.

3.0 MATERIALS AND METHODS

3.1 Study area and sampling

Samples of water, sediment and fishes were taken from the aquaculture pond at 7^{th}, 6^{th}, 5^{th} miles of Kuching Serian Road and Semariang Batu. At 7^{th} and 5^{th} miles, three types of samples which were water, sediment and fish were taken. As for Semariang Batu and 6^{th} miles, only water and fish were collected. For the water and the sediment, samples were taken from three different spot of the pond whereas fish samples were taken from only one spot. The samples were transported to the laboratory in ice container.

3.2 Sample Processing

The samples were processed immediately upon arrival in the Microbiology Laboratory, Department of Molecular Biology, Faculty of Resource Science and Technology, UNIMAS. All the samples were serially diluted in sterile test tube using 8% saline solution as medium. 100 µl of 10^{-1}, 10^{-2}, and 10^{-3} dilutions were spread onto EMB (Eosine Methylene Blue) agar in duplicates. 1 ml of water samples, 1 gram each of the sediments and intestines were diluted with saline before they were spread on the EMB agar. The plates were incubated at 35^{0}C for 24 hours.

3.3 Isolation of *E. coli* from samples

Colonies showing metallic green sheen colour on the EMB agar were presumed as *E. coli* and at least three colonies were picked from the colonies agar plates. Those colonies were restreaked on new EMB agar and incubated at 35^0C for 24 hours to produce pure culture. Further confirmations of the isolates were carried out by conventional laboratory methods including biochemical test, gram staining and colony morphology.

3.4 Biochemical Test for confirmation of *E. coli*

Biochemical tests were conducted for further confirmation of the isolates including indole test, motility test, sulphide production test, methyl red test, Voges- Proskauer test and citrate utilization test. *E. coli* ATCC 25922 serves as a reference strains for the entire biochemical test that have been conducted.

Indole test, motility test and sulphide production test were conducted according to PML Microbiological Technical Data Sheet, (2001). Isolates were stabbed at the centre of the SIM medium using the straight inoculating needle. The tubes were incubated with loosen caps at 35 ^{0}C for about 18-24 hours. H_2S and motility tests must be interpreted before adding the Kovac's reagents. Turbidity or fuzzy growth away from the line of inoculation denotes motility. Growth only along the stab line indicates negative results for the motility test. Blackening of the medium indicates positive results, while absence of the blackening indicates the negative results of the H_2S production. A few drops of Kovac's reagents were added into the medium and the colour changes were observed for the indole test. A pink red

colour indicates positive results while yellow colour denotes negative results for the indole test.

Methyl red and Voges-Proskauer tests were conducted according to PML Microbiological Technical Data Sheet, (2001). Samples were inoculated in the MRVP medium and incubated in the incubator at 35^0C for 48 hours. The broth was aliquot (1 ml) aseptically into another sterile universal bottle for VP test. Five drops of methyl red indicator were added to an aliquot of the broth. The results were indicated by the immediate colour changes of the broth. Red colour of the medium indicates positive result while no colour changes indicates negative result. Few drops of Barrit A followed by Barrit B reagents were added into the broth culture. Results were determined after 15 minutes. Positive result is indicated by the development of a distinct red color while negative result produced no colour changes (yellow).

Citrate utilization test was conducted according to Sridhar, (2006). Isolates were inoculated onto slope of Simmon's citrate agar and incubated at 37 ^{0}C for 24 hours. Positive results were indicated when the colour has changed from green to blue and if the colour remains green, it indicated negative results.

3.5 Antibiotic Susceptibility Test

Antibiotic susceptibility test were performed by the disk diffusion method on Mueller Hinton agar according to National Committee for Clinical Laboratory Standards (NCCLS) (1993). Isolates were tested with eight different types of antibiotics, which were chloramphenicol (30µg), erytromycin (15µg),norfloxacin (10µg),nitrofurantoin (300µg), tetracycline (30µg), ampicillin (10µg), nalidixic acid (30µg) and carbenicillin (100µg).

Single colonies of *E. coli* were picked up with a loop and transferred into the LB broth. The broth cultures were incubated at 35 ^{0}C for 24 hours until it achieved or exceeds the turbidity of 0.5 Mc Farland standards. The turbidity was adjusted by adding broth or adding isolates to obtain the turbidity optically comparable to that 0.5 Mc Farland. Within 15 minutes after adjusting the turbidity of the inoculum suspension, the inoculum was inoculated on the Mueller Hinton Agar surface using a sterile cotton swab. The procedure was repeated by streaking two more times while rotating the plates 60^0 each times to ensure even distribution of the inoculum.

The antimicrobial discs were dispensed on the inoculated agar plate using disc dispenser. The plates were inverted and placed in an incubator at 35^0C for 16 to 18 hours. The diameters of the zone of complete inhibition were measured. Zones were measured to the nearest whole millimeter using a ruler. The sizes of the zones of inhibition of ATCC 25922 are interpreted by referring to table 1a (Appendix 1,Acceptable Limits for Quality Control Strains Used to Monitor Accuracy, Nonfastidious Organisms Using Mueller Hinton Medium Without Blood or Other Supplement) and for the isolates by referring to table 1b (Appendix 2, Zone Diameter and Minimum Inhibitory Concentration Interpretive Standards for *Enterobacteriaceae*) of the CLSI M02-A10: Performance Standards for Antimicrobial Disk Susceptibility Tests: Tenth Edition and the organisms were reported as resistant, intermediate or susceptible.

3.6 Crude DNA preparation by Boiled Cell Method

DNA from the isolates was extracted by boiled cell method as described by Bilung *et al*., (2005). A colony was picked from the nutrient agar and inoculated into 5 ml of LB broth

and grown for 24 hours with shaking at 120 rpm at 37 ^{0}C. From the LB broth culture, 1 ml was spun at 10, 000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1 ml sterile distilled water and was boiled for 10 minutes. The tube was placed on ice immediately for 10 minutes followed by spun it at 10,000 rpm for 5 minutes. The clear supernatant (20 µl) was transferred into a new tube and 5 µl was used for PCR analysis.

3.7 RAPD-PCR Analysis

PCR based fingerprinting was carried out according to method described by Radu *et al*. (2000). Gen1-50-09 (5'-AGAAGCGATG-3') and Gen1-50-10 (5'-CCATTTACGC-3') were used as primers for the RAPD analysis.

PCR- based fingerprinting was carried out in a volume of 25µl containing 20 to 40 ng (5µl) of *E. coli* DNA template, 2.5mM $MgCl_2$, 25pmol of primer, 2.5 U of Taq DNA polymerase, 1mM dNTPs mixture and 10X reaction buffer. PCR reactions were performed on a Swift miniPro Thermal Cyclers under the following conditions: 35 cycles of 94^{0}C for 1 min, 36^{0}C for 1 min and 72^{0}C for 2 min, and 1 cycle of final extension of 72^{0}C for 5 min.

The exact volume of each ingredients used for a PCR reaction mixture and the parameter for the PCR reaction are summarized in Table 2a and Table 2b, respectively. A negative control without DNA was included in each RAPD run.

3.8: Agarose Gel preparation and Agarose Gel Electrophoresis

A 1% agarose gel was prepared by heating 0.5g of agarose powder in 50ml 1X TBE buffer in the Erlenmeyer flask and then melted in a microwave oven for a few minutes. The solution was allowed to cooled and poured into a gel mold with the combs positioned in it.

The gel was left to solidify and the comb was removed with care. The tank was then filled with 1X TBE buffer until the gel was covered by the buffer.

Five microlitres of the DNA template was mixed with 1 µl of gel loading dye by mixing them on the parafilm. Six microlitres of the PCR product were separated on a 1% agarose gel. The DNA ladder (6 µl) and the samples were loaded into the appropriate well, respectively. The cover was placed on the electrode box and the wire was plugged in. Gel electrophoresis was performed at 100 volts for 60 minutes. After the electrophoresis, the gel was stained with Ethidium Bromide and was visualized on the UV traansilluminator. The gel was photographed using AlphaDigi Doc RT.

3.9 RAPD-PCR Fingerprint Analysis

The profiles of the DNA bands obtained with the RAPD-PCR were analyzed with RAPDistance Package Version 1.4 (Amstrong *et al.*, 1996). The bands patterns obtained from the gels were scored in the binary data format for each of the primer used. The scoring was made based on the presence or absence of the bands. The distance calculator of similarity bands profiles was based on the Mathematical model for studying genetic variation (Nei e*t al.*, 1979).

Table 2a: Contents of PCR mixture

Solution	Final Concentration	Volume(µl)
$MgCl_2$	25mM	1.5
PCR Buffer	10X	2.5
dNTPs mixture	0.5mM	0.5
Primer	2.5pmol/ µl	1.0
Taq DNA Polymerase	2.5U/µl	0.5
Template DNA	20-40ng	5.0
Distilled Water	-	14
Final Volume:		25

Table 2b: Cycle profiles of PCR

Condition	Temperature(^{0}C)	Time(minutes)	Cycles
Initial denaturation	94	1	1
Denaturation	94	1	35
Annealing	36	1	
Extension	72	2	
Final extention	72	5	1

4.0 RESULTS

4.1 Biochemical Test for Confirmation of *E. coli*

Samples of water, sediment and fish were analyzed for the presence of *E. coli* by plating on EMB agar. Out of 69 isolates of suspected *E. coli*, 19 isolates (27.54%) showing metallic green sheen colour on EMB agar, (Figure 1) were confirmed by their morphology and biochemical tests. The 19 isolates (27.54%) were positive for the presence of *E. coli* based on the IMViC test and other biochemical test. The isolates designation, their source of isolation and their origin of sample are shown in Table 3.

Table 3: *E. coli* strains

***E. coli* isolates**	**Source**	**Location**
B5-1-W-5	Water	5th mile
B5-1-S-4	Sediment	
B6-1-W-6	Water	6th mile
B6-1-F-1	Fish	
B6-1-F-2	Fish	
B6-1-W-8	Water	
B6-1-W-7	Water	
B6-1-W-4	Water	
B6-1-W-5	Water	
B6-1-F-3	Fish	
B6-1-W-10	Water	
B7-2-S-BC4	Sediment	7th mile
B7-1-F-2	Fish	
B7-1-F-3	Fish	
B7-2-S-BC6	Sediment	
SB-1-W-1	Water	Semariang Batu
SB-1-F-1	Fish	
SB-1-F-3	Fish	
SB-1-F-2	Fish	