CHAPTER 1

INTRODUCTION

Estuaries are one of the most productive ecosystems in the world. Many animal species rely on estuaries for food and as places to nest and breed. Estuaries trap nutrients and sediment that are carried from the land by rivers and from the ocean by tides. In this modern day, coastal water bodies including estuaries play an increasing role in sustaining various human economical activities, such as human settlement, aquaculture, waterways and harbour. The rapid development and uncontrolled use of estuaries have caused various public health issues such as human pathogens contamination of the water bodies. The microbiological contamination of waterways by pathogenic microorganisms is one of the most important public safety concerns in the world (Yan and Sadowsky, 2007). Concern on the water quality is increasing in the recent years due to frequent detection of pathogenic bacteria in the aquatic systems (Meays *et al*., 2004). Kuala Sepetang estuary is one of the areas that provide commercial economic benefits to the Malaysian. It is located on the west coast of peninsular in the state of Perak, Malaysia, supports a wide range of aquaculture products due to its warm tropical weather and healthy mangroves providing protective nursery grounds for fishes, crustaceans and mollusks.

Microbiological quality and safety monitoring of water bodies, specifically rivers and estuaries, is an essential component of national risk management program in most countries. During the past decades of development, the microbiological quality of water based on culture-dependent methods which optimized continuously to detect the presence of relevant organisms, public health and risk assessment studies (Leclerc, 1994; Leclerc and Moreau, 2002; Mossel and Struijk, 2004). Culture-dependent methods has been improved in recent decades for the detection and quantification of

fecal indicator bacteria in waters, which include the most commonly used, coliform and *E. coli,* and more recently, *Enterococci*. Whether it is based on MPN, direct culturing or membrane filtration approaches, selective and/or differential media is needed to quantify the relevant organisms. For example, quantification of coliform and *E. coli* can be accomplished by using Endo agar and Colilert system (IDEXX Laboratories, Westbrook, Maine); while *Enterococci* could be detected with mEndo medium. Unfortunately, no one single medium is able to perform quantification or detection of coliform, *E. coli* and *Enteroccoci* simultaneously. At least two different culture media are needed to quantify to detect these organisms, and this is time consuming and costly. In addition, detection of common waterborne pathogens in waters requires specific culture medium too, and similarly, no one single medium could accurately detect the presence of multiple types of potential pathogens in the waters. Hence, quantification of fecal indicators is commonly used in monitoring of water quality and safety instead of direct detection of the relevant waterborne pathogens of concern. The work load, time and cost needed to detect all of the pathogens of concern in a long term national monitoring is simply not economically feasible.

The quest to overcome the bottleneck in microbial water analysis has provided the impetus to develop culture-independent methods. Bacterial diversity study of river and coastal environments is essential to determine the ecology and evolution of bacteria, and it also plays a main role in supporting management policies or sustaining risk assessment studies. Immense diversity of uncultured organisms has been revealed by culture-independent methods. Moreover it attracts the attention to implement complementary approaches to the analysis of water bacterial diversity (Amann *et al*., 1995; Hugenholtz, 2002; Kemp and Aller, 2004; Alain and Querellou, 2009). Several scientific and technological developments, including the inexpensiveness and rapidity of culture-independent methods, particularly PCR method, have brought obvious

improvements to bacterial diversity studies. However, cultivation methods are still fundamental to monitor water quality control and also to make the inferences on the physiological and metabolic properties in the organisms (Palleroni, 1997; Cardenas and Tiedje, 2008). On the other hand, bacterial diversity study of river and coastal environments is essential to assess the ecology and evolution of bacteria to support management policies or to sustain risk assessment studies. Molecular methods, particularly PCR based method, such as REP-PCR have provided a rapid way to study the bacterial genomic diversity. REP-PCR has been suggested as promising analytical tool to study the genomic diversity of *E. coli* in water (Somarelli *et al*., 2007).

Thus, in this study, the suitability of a multi-chromogenic media, CHROMagar™ Orientation, which is commonly used in clinical diagnostics for rapid isolation of a wide range of Gram-negative and Gram-positive bacteria, was explored for application in bacterial diversity study and waterborne pathogens monitoring in natural waters.

The main objectives of this work were:

a) To evaluate whether of CHROMagar™ Orientation can be used as the sole medium to identify the pathogens and coliform bacteria from estuaries

b) To comparative the abundance of human-associated bacterial pathogens in the surface waters and sediments along Kuala Sepetang estuary

c) To investigate the genetic relatedness of estuarine *Escherichia coli* isolated along Kuala Sepetang estuary using REP-PCR.

CHAPTER 2

LITERATURE REVIEW

2.1.Estuary

Estuaries house a variety of birds, fish and animals. It serves as food and refuge source and also provides harbour for many plant species and other organisms (NOAA, 2012). Estuaries are crucial coastal and marine habitat because they are one of the most productive areas on earth due to providing the rich feeding ground some wild animal like birds and breeding ground for other marine life like fish, and providing a coastal area culture such as recreation and education and they help in regulation the natural systems, so all these make estuaries as important sites for human settlement and use (Environment Canada, 1987). However, uncontrolled human activities have made estuaries as one of the most threaten ecosystem on the earth.

Estuary is semi enclosed water body forming when fresh water from river joins with salt water of the ocean. This mixing of water creates a transition zone which is known as an estuary. These waters mixing from different sources which are different in salt concentrations, river or fresh water with low salt concentration and ocean with the high salt concentration provide brackish water in estuary area. Estuary is strongly affected by tides and tidal cycles. Many estuaries are protected from the full force of ocean waves, winds, and storms by reefs, barrier islands, or fingers of land, mud, or sand that surround them. The characteristic of each estuary depends upon the local climate, freshwater input, tidal patterns, and currents (NOAA, 2012). Estuaries provide various unique habitats that support highly diverse communities and provide crucial links to nearby ecosystems (Mclusky and Elliott, 2004).

Rapid growth and uncontrolled development in many coastal areas in all over the world has resulted in deteriorating of estuaries. It is frequently contaminated with industrial, agricultural and anthropology wastes. Many of these contaminants such as bacteria and chemical materials are known to concentrate in water or sediments in estuaries. (McCain *et al*., 1988). A main emphasis about doing research in the estuary is to study the pollution effects in estuaries. The major pollution in estuary is caused by heavy metal and microorganism contamination (Kennish, 2002). The concerns about estuaries pollution started since 1960s and is predicted that scientific work related to estuaries pollution would still grow rapidly in future (Sun *et al*., 2012).

As examples of water contamination or bacterial pathogens in water, *Vibrio Cholera*, *Salmonella*, *Shigella* and *E. coli* are most important bacterial transmitted through water and can cause bacterial diseases Cholera, typhoid fever, bacillary dysentery and diarra, respectively (Cabral, 2010) In some study estuaries were evaluated due to chemical contamination, biomarker was used as an indicator for chemical contamination and its effect on organismal level (Hugget *et al*.,1992).

2.2.Microbiology quality and safety in estuaries

Nowadays, more than half of the nation's population live in coastal communities. Therefore their activities could increase the microbial contamination in the estuaries, rivers and ocean, which eventually lead to public health threat due to waterborne diseases (Nadakavukaren 2000; USEPA 2000; 2005; SCDHEC, 2007). There are many sources that contribute microbial contamination to the coastal environment, including domestic pets, livestock, wildlife and humans (USEPA, 2005; Kelsey, 2006).

According to Clean Water Act 303(d) reports, bacteria is a primary contaminant in all water bodies and can cause a significant threat to human health even at very low concentrations (USEPA, 2000; Simpson *et al*., 2002). Many of the human pathogen, such as *E. coli, Enterococos* sp., *Salmonella* sp., and *Cryptosporidium* sp. are introduced into the water via fecal contamination (Guan *et al*., 2002; Field *et al*., 2003; Meays et al., 2004). Thus, monitoring of water quality is carried out by enumeration of fecal indicators in the water to indicate the presence or absence of dangerous human pathogens (USEPA, 1986; Schueler and Holland et al., 2000; Anderson et al., 2005). Detection of various types of human pathogens in the water is too tedious and costly to perform.

2.3.Fecal indicator in water

Detection of various human pathogens presence in the water is non-practical. Since most of the human pathogens are introduced into the water via fecal contamination, a measure is required to monitor or alert the present of pathogens in water resulting of fecal contamination (NHMRC, 2003). At least 500-1000 various species of bacteria exist in human gastrointestinal which can be transmitted to the coastal and fresh water. There are usually 10 to 20 genera predominate (Table 2. 1) (Cabral, 2010).

Table 2.1. Total viable count (Log10 CFU/g) of predominant microbial genera in feces of healthy human.

Microbial group	Log_{10} CFU/g feces
Bacteroides	11.3*
Eubacterium	10.7*
Bifidobacterium	10.2*
Ruminococcus	10.2*
Peptostreptococcus	10.1*
Peptococcus	10.0*
Clostridium	9.8*
Lactobacillus	9.6*
Propionobacterium	9.4*
Actinomyces	9.2*
Methanobrevibacter	8.8*
Desulphovibrio	8.4*
Fusobacterium	8.4*
Enterococci	3.5–7.2**
Enterobacteriaceae	5.9–8.0**
Escherichia coli	7.5–7.7**
Citrobacter	3.3**
Klebsiella	2.4**
Yeasts	1.0–2.5**

*Values expressed as dry weight.

**Value expressed as wet weight

(Source: Cabral, 2010)

An indicator should be easy to measure so as to alert the present of fecal pathogens in water (NHMRC, 1996; WHO, 1996). Coliforms are used as bacterial indicators to monitor the water quality because they can be identified easily, do not grow in nature water and present in high number in human and animal feces. Coliforms are not considered as pathogens except for a few exceptions but their presence shows that fecal contamination may have occurred and resulting in the present of pathogens in water. For more than 100 years *E. coli* and coliform are being tested for indication of fecal

pollution in monitoring of the public health risk in water (NHMRC, 2003). *Enterococci* are other fecal bacteria which are being used as fecal indicator bacteria. Using of *Enterococci* as fecal indicator was suggested in Europe since 1998 due to limitation of total coliforms. However many alternative indicator to total coliforms and *Enterococci* have been proposed including of *Bacteroides fragilis*, *Bifidobacteria,* bacteriophages, sulfite-reducing clostridia, and nonmicrobial indicators such as faecal sterols (WHO, 1996; NHMRC, 2003).

2.3.1. Total colifrom

The total coliform group belongs to the family of *Enterobacteriaceae* including Gram-negative, aerobic and facultative, non-spore-forming and rod-shaped bacteria that produce gas in lactose fermentation within 48 hours at 35-37℃. Total coliform is a group of several species of bacteria with similar characteristics such as *Klebsiella, Enterobacter, E. coli, Hafnia, Serratia, Morganella* and *Citrobacter*. Although they are found in high number in human and animal feces, not all of them are fecal origin. They are useful to monitor the quality of water, recreational waters and shellfish harvesting waters. They are less sensitive compared to viruses and protozoan cysts in different environmental conditions. Some member of coliform such as *Klebsiella* sp can be environmental origin (Gabriel, 2005).

2.3.2. Fecal coliform

Fecal coliform are thermotolerant bacteria that can ferment lactose in higher temperature. This group comprise *Klebsiella, Enterobacter, E. coli* and *Citrobacter* (Gabriel, 2005). *E. coli* comprises the majority of thermotolerant coliform in drinking

water. The presence of thermotolerant coliforms is correlated with the presence of enteric pathogens in environment. In general this group is reliable as indicators for disease-causing bacteria (EPA, 2006). This group of bacterial less effective to determine the present or absent of viral or protozoan in aquatic environment compared to bacteria (EPA, 2006; Gabriel, 2005). *E. coli* growth detection in pristine site in tropical rain forest suggests that using *E. coli* as indictor of fecal contamination in tropical may not be reliable (Hazen et al., 1988). The thermo-tolerant coliforms are more specific indicator of fecal contamination compared to total coliform because most of them are associated with the recent fecal contamination (EPA, 2006).

2.3.3. *Escherichia coli* (*E. coli*)

Hundred years ago scientists found that human faeces contained bacteria if it present in water, showed that the water was not safe to drink. In 1885 Escherich discovered a bacterium present in faeces, which he named Bacterium coli (*B. coli*) and it is now called *Escherichia coli.* The concept that the presence of *B. coli* in water caused water pollution was readily adopted. It is also reported that the concept of indicators had been suggested by van Fritsch considering to his observation of *Klebsiellae* in humans faeces which were also present in water (Hendricks et. al., 1978). At the beginning it was difficult to differentiate *B. coli* from other coliform bacteria in faeces and water, thus many methods and techniques were developed and carried out to recovery of coliform bacteria, and several analyses were used to confirm if any of the recovered coliforms were *B. coli*. Water bacteriologist for the next 50 years focused on obtaining these techniques to approve the present of *B. coli* in water and show it apart from other intestinal bacteria. In the 20th century, methods were available which could differentiate

B. coli from other bacteria. The methods founded in the late 1800s and early 1900s are still used to monitor and determine of faecal pollution in water (NHMRC, 2003).

2.3.4. Enterococci

To improve the monitoring of water quality, especially during monitoring of fecal contamination, *Enterococci* analysis has been used. *Enterococci* is Gram-positive *cocci*, facultative anaerobic spore forming and tolerant to wide range of environmental condition such pH sodium chloride. This group of bacteria is found in intestinal tract of humans and animals. *Enterococci* does not grow in environment except in tropical environment and *Enterococci* is alternative to coliform regarding to water quality indicator (Gleeson and Gray, 1997). *Enterococci* has some advantages as a microbial indicator compared to coliform, fecal coliform even *E. coli*, because it does not grow in environment. It means this group of bacteria is generally absent in waters having no connection with human and animal life and they survive longer in environment. Although they are present in low compared to coliforms and fecal coliforms in human faeces, they are still numerous enough to be detected after dilution (APE, 2006; NHMRC, 2003).

2.3.5 .Other fecal indicator in water

E. coli and *Enterococci* are the main key faecal indicators bacteria to monitor the contamination water (NHMRC, 2003). *Clostridium perfringens* are Gram-positive, rod shape and anaerobic bacteria and they are wieldy distributed in nature and have been isolate from animal intestinal. Using of *Clostridium perfringens* as faecal indicator was first suggested in 1899 (Gleeson and Gray, 1997). Bacteriophages are viruses which infect the bacteria. They have been suggested as microbial indicator as their behaviour

is more like human enteric viruses which pose a health risk to water consumers if water has been contaminated with human faeces (NHMRC, 2003).

2.4. Waterborne pathogens

Microorganisms that can cause disease in host are called pathogen. Host can be human, animal, plant even other microorganisms. Pathogens that can be contributed in water and cause disease comprise bacteria, protozoa and viruses. Waterborne pathogens are linked to those fecal microbes when released in to the water and they have the ability to cause different type of diseases. They are connected to foodborne transmission via filter feeders such as shellfish or those foods that washed or prepared by contaminated water. Enteric illness comprise a variety of different clinical symptoms are not limited just to diarrahea, nausea, vomiting and abdominal pain (Moe, 1996). Water borne pathogens can be deadly or cause very dangerous illness. Waterborne pathogen agents are transmitted to human and animal through contaminated water. The sources of water contamination are the excreta of infected humans and animals.

2.4.1. *Escherichia coli*

E. coli was described by Theoder Escherich in 1885. *E. coli* is Gram-negative, rod shaped bacilli, lactose positive, mostly non-pathogenic, motile by peritrichous flagella. *E. coli* is a member of *Enterobacteriaceae* family which is present as normal flora in the lower intestine of both humans and animals (Ewing, 1986; Weintraub, 2007). Different kinds of fimbriae are produced by *E. coli* which is important during the adhesion to the host cells (Percival *et al*., 2004). It is the most predominant facultative anaerobic bacteria in humans and most animal’s colonic flora. *E. coli* colonizes the

infant's gastrointestinal tract within hours of birth, which is arriving with foods or the individuals handling child (Percival *et al*., 2004; Weintraub, 2007).

E. coli is the first facultative organism of the human gastrointestinal tract. If these bacteria do not acquire any genetic elements which is coding virulence factor they will remain harmless. Those *E. coli* strains which acquire virulence factor, "foreign DNA" encoding enterotoxin, adhesions or invasion factors become virulence and can cause diseases (Bell, 1998). Pathogenic strains cause diarrhae in healthy adult humans. However, virulent strain like O157 can cause very serious illness even can cause death.

Some of them are useful for body by their help to synthesizing of vitamins and suppression the harmful bacteria. Different strain of *E. coli* has specific host, it make possible to determine the source of fecal contamination in environmental samples (Sussman, 1985). Depending on which type of *E. coli* strains may present in water and sediment samples, it can be assumed that origin of contamination is whether from human, animal source.

The enterotoxin secreting by *E. coli* was identified by De *et al* (1956), it causes diarrhea or intestinal diseases. The intestinal diseases causing by *E. coli* were classified into six groups based on virulence properties of *E. coli,* they are different in O: H serotypes and epidemiology (Nataro *et al*., 1998; Weintraub, 2007). These six groups are Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC) or Verocytotoxigenic *E. coli* (VTEC), Enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Weintraub, 2007).

ETEC strains contain specific plasmid which produce enterotoxins and can cause diarrhea after attachment to their receptors on intestinal epithelium. This enterotoxins are classified into two main class, (I) heat-labile (LT) enterotoxin and (II)

heat-stable (ST) enterotoxin. LT is divided into two type LT1, which is pathogenic for both human and animal and LT2, mostly identified in animal *E. coli* isolates but there is no associated with causing dieses in both human and animal. There are two major subtypes for ST, STa, other than *E. coli, Yersinia enterocolitica* and *V.cholerae* non-O1 can produce this enterotoxin and STb, this enterotoxin found only in *E. coli* (ETEC) (Levine, 1987). This strain first was recognized as diarrhea cause in piglets. The first descriptions of ETEC in human reported when ETEC strains were isolated from children stools (Taylor, 1961).

E. coli (VTEC) strains were identified by Konowalchuk *et al*. (1977). This strain produces a toxin and has irreversible effect on vero cells. This toxin is just sytotoxin for vero cells. The other important *E. coli* strain is EHEC. There are more than 200 serogroups of EHEC which are producing shiga-toxins (STX). *E. coli O157:H7* is the most important strain in this group which cause haemolytic uraemic syndrome (HUS), especially in children, thorombocytopaenic (TTP) and death (Coia 1998; Callaway TR 2003; Dundas S 1999). *E. coli* (EPEC) is diffusion strain attach to HEp-2 cells can cause diarrhea but without any effacing lesion. EAEC is aggressive strain and attach to HEp-2 cells. It induces shortening of the villa in intestinal cause mocoid diarrhea without vomiting (Weintraub, 2007).

2.4.2. ***Salmonella***

Salmonella are Gram negative, facultative anaerobic, flagellated bacteria consisting of non-spore forming bacilli and a member of *Enterobacteriaceae* family (Robles *et al*., 2009). There are 2 species of *Salmonella, S.bongori* which is rarely related to human infection and *S. enteric. S. enteric* including the other reminding subspecies such as *S. enterica* serovar *Typhimurium, Enteritidis* and *Typhi* (Dunkley *et al*., 2009). The four

most common *salmonella* which are causing human infectious are *Typhimurium, Enteritis, Newport*, and *Heidelberg* (PFD, 2000; CDC, 2003). These four serotypes were also the most frequently isolated from food samples. Salmonellosis caused by species in the genus *Salmonella* was described in 1984 as a new and significant threat to the public health by World Health Organization (FAO 1984; Robles *et al*., 2009). Salmonella can multiply in environment rather than intestinal. In environment, Salmonella tends to from biofilm on both organic and inert surfaces. Thus, bacteria are better protected against environmental stresses. Salmonellosis infectious is caused via contaminated water or food/feed (Lunestad *et al*, 2007).

2.4.3. *Vibrio*

Genus *Vibrio* are Gram-negative, non-spore forming bacilli, facultative anaerobic, oxidase positive, halophilic and motile by means of a single, sheathed, polar flagellum bacteria which are native to marine and estuarine waters. *Vibrio* genus member are endemic to coastal and estuarine systems around the world, and this group includes several species with important human health impacts. The most medical significance, *Virbrio* species are *Vibrio cholerae, Vibrio vulnificus,* and *Vibrio parahaemolyticus.* (Jennifer, 2007).

Vibrio cholerae is a well-known human pathogen that has caused cholerae epidemics all over the world in many developing countries (Villalpando, 2000). *V. cholerae* can threat public health when it is ingested via untreated water, contaminated seafood (raw or under-cooked), or exposure of skin wounds to sea water (Whittman and Flick, 1996; Potasman *et al*., 2002; Scott *et al*., 2002). There are two important serotypes of *V. Cholera: O1* and *O139. O1* is the responsible for mostly happened cholera epidemics in the world. Infection due to *V. cholerae* begins with the ingestion of

contaminated water or food. The subsequent loss of water and electrolytes leads to the severe diarrhoea characteristic of cholera. *V. cholerae* remains a major threat in many places around the globe as the causative agent of cholera, and its reservoir in the estuarine environment may play an important role in the survival and transport of pathogenic strains (Hisieh *et al*., 2007).

Vibrio vulnificus is very important pathogenic *Vibrio* because of its invasiveness and the high fatality rates associated with infection. It was first identified and described by the CDC in 1976 and has become the leading cause of seafood-associated deaths in the world. *V. vulnificus* is a halophilic (salt-loving) organism which its infectious dose is unknown. It can also cause an infection of the skin when open wounds are exposed to warm seawater. One of the most deadly food-borne pathogens, *V. vulnificus*, is the probable cause of the greatest number of seafood-related fatalities in the United States (Hisieh *et al*, 2007).

Vibrio parahaemolyticus is a common bacterium in marine and estuarine environments. This organism is recognized as a major, worldwide cause of gastroenteritis, particularly in areas of the world where seafood consumption is high such as Southeast Asia (Joseph *et al*., 1982). It is an emerging pathogen in North America (McCarter, 1999). The most common clinical manifestation of *V. parahaemolyticus* infection is gastroenteritis (Levine, 1993). It is less deadly, but causes a greater number of food-borne illnesses and can result in pandemic outbreaks of disease (Hisieh *et al*., 2007).

Vibrio sp., are one of the most important waterborne pathogens. They are responsible for twelve outbreaks in U. S. in last 30 years (Potasman *et al*. 2002). This bacterium is very difficult to control in marine and estuarine environments due to being autochthonous to these environments.

2.4.4. *Aeromonas*

Aeromonas is Gram-negative, facultative anaerobic rod form which morphologically resembles as member of *Enterobacteriaceae* family. *Aeromonas* species are recognized etiological agent for wide range of diseases in human and animal (Janda and Abbott 1998). In developing countries, the potential of pathogenic *Aeromonas* sp., are very common in drinking water and food, especially sea food. There are several waterborne and foodborne outbreaks related with *Aeromonas* have been reported. These organisms are important due to causing of skin and soft-tissue infection. *Aeromonas* sp., have been found to be antimicrobial resistance bacteria (including third-generation cephalosporin and the fluoroquinolones), especially clinical source from some developing country in Asia (Ghenghesh *et al*, 2008). The large majority of *Aeromonas* are caused by *A. hydrophila, A. Caviae* and *A. Veroni biovar sobria. Aeromonas* sp., are primarily aquatic organisms in different freshwater bodies including river, lake and streams. They are also predominant in estuarine water and can be easily isolate from surface water of sea. They can be found in raw sewage and activated sludge. These microorganisms can survive in high salinity water (Hazen *el at*, 1987; Ghenghesh *et al*, 2008).

2.4.5. Other waterborne pathogen

Major waterborne pathogen agents are *Vibrio cholera* responsible for cholera, *Salmonella* and *E. coli 0156:H7* which is causing dysentery cases in both developed and developing countries. There are some other bacteria considering as waterborne pathogens rather than *Vibrio, Salmonella* and *E. coli O157,* include *Campylobacter* sp*., Shigella* sp., *Staphylococcus* sp., *Bacillus* sp., and *Pseudomonas* sp., which have caused various diseases and outbreak (Ghinsberg *et al*., 1994; Rippey, 1994). Total coliforms,

Fecal coliforms and *Enterococus* are other important bacteria that can cause disease via water and they are being used to monitor the water quality. (Slanetz and Bartley, 1957).

Pseudomonas spp., are other important waterborne pathogens causing diseases in humans (Ghinsberg *et al*., 1994). There are some other pathogens like *Acinetobacter* sp., which can be found in groundwater, surface water, sewage water, drinking water. These bacteria have been isolated in 97% of natural water (WHO, 2006).

The genus *Exiguobacterium* is a newly described bacterium by Collins *et al*. (1983). This bacterium is a Gram-positive marine bacterium which can cause infectious disease and it may be difficult to detect by normal laboratory test, to identify this bacterium molecular technique should be applied (Keynan *et al*., 2007). *Stenotrophomonas* sp., are another bacteria which can be found in marine and mangrove environments but this bacterium is more related to the plant. Fatal infections in human can be occurred due to *Stenotrophomonas* sp infection (Ryan and Monchy, 2009). In general 90% of bacteria in marine environment are Gram-negative and most of the Gram-positive in seawater belong to *Bacillus* sp., (Nerkurkar *et al*., 2009).

2.5.Sediment

Estuaries floor is covered by layer of sediment and it acts as sink for both marine and fresh water. The presence of fecal indicator bacteria (FIB) in sediment represents a risk for human health as they show the presence of other pathogenic organism (An *et al*., 2002). The coastal sediment can act as reservoirs for pathogenic organism. The estimation of water quality in coastal area is undertaken by the enumeration of fecal coliforms and *E. coli* from the water. However the studies shows that the number of

fecal coliform and *E. coli* in sediment can be 10-10000 fold greater than in the water (Craig *et al*., 2002).

Some studies showed that sediment characteristics, like organic carbon, nitrogen content and practical size, correlate with number of bacteria. In coastal water the risk of human infection may be increased due to re-suspension of pathogenic organism from the sediment surface during recreational activities. One of the important problems during the enumeration and detection of bacteria from the sediment is their attachment to sediment particles (McDaniel and Capone, 1985). The studies showed that the large population of bacteria present at the surface of the sediment and it has been confirmed that the population of bacteria generally is decreasing with increasing the deep (Parkes *et al*., 2000). Various different types of pathogenic bacteria, such as coliforms, *E. coli, Salmonella sp., Shigella* sp, *Pseudomonas* sp. and *Vibrio* sp., can be present in sediment. The diversity of bacteria in sediment depends on sediment characterization which effects on the presence and survival of bacteria. Gerba and McLeod (1976) showed that *E. coli* and *Enterobacter* survive in estuarine sediment for longer time compared to seawater because the estuarine sediments are more enrich due to organic matter (Gerba and McLeod, 1976; Burton *et al*., 1987).

2.6.Culture-dependent and culture-independent method

2.6.1. Culture-dependent method

Traditional microbiology research that is carried out in the field on bio-deterioration was based on cultivation methods. Cultural based methods extremity useful to understand the physiological isolated organisms. The results obtain by using this technique are covered those microorganisms which could be cultivated (Amann *et al*., 1995; Ward *et al*., 1990).

There are some new culture media have recently developed which are encouraged due to their advantages of having pure isolate and function of physiological and metabolic studies. Chromogenic media are one of the important new generations of media which are being used in microbiological studies.

2.6.1.1.Chromogenic Media

Chromogenic and flourogenic media are microbiological growth media comprising chromogenic, fluorogenic substrates or a combination of both, which are used as powerful tools in detection of microorganisms due to detection of specific enzyme which is produced by interested microorganism. These substrates can be ONPG (ortho-Nitrophenyl-β-galactoside) and MUG (Methylumbelliferyl-b-D-glucuronide), and they are organism specific. The target microorganisms are characterized by their enzyme systems which metabolize the substrate and release the chromogen of fluorgen, resulting in different coloration or fluorescence change of certain bacteria colonies. So, chromogenic media can enhance the bacterial identification over than conventional culture media base on color reaction. Thus, this specificity and intensity of chromogenic media can optimise the enumeration of microbial resulting easy and rapid detection of bacterial colonies (Merlion *et al*., 1996; Conda, 1960).

Chromogenic media are being widely used, especially in clinical laboratory to detect and identification of pathogenic bacteria (Sharmin, 2010). Urinary tract pathogens are important and they cause relatively common diseases. Thus, many studies have been done due to using chromogenic media for rapid detection and identification of urine tract pathogens mainly *E. coli, Klebsiella, Enterobacter* and other *Enterobacteriaceae* family (Sharmin, 2010; Merlion *et al*., 1996). There are different types of cheomogenic media, CHROMagar^{TM} Orientation is non selective and differentiation medium which is

supporting the growth of all pathogens (Gram-negative and Gram-positive bacteria) unlike the MacCokey agar medium (Sharmin, 2010).

Using of CHROMagar^{TM} Orientation media has been increased, especially in clinical laboratory to detect some bacteria rather than *Enterobacteriaceae* such as MRSA/ORSA. This medium has been evaluated for detection and identification of Gram-negative and Gram-positive pathogenic bacteria (Samra *et al*., 1998; Merlion *et al*., 1996). The result showed that this medium can be used as rapid culturing method for presumptive detection of various types of pathogenic bacteria (Samra *et al*., 1998; Merlion *et al*., 1996).

In recent years, rapid identification of these indicator microorganisms has been improved by using chromogenic media (Baylis and Patrick, 1999). Various selective and differentiated chromogenic media are available which can be used to detect different type of bacteria, such as CHROMagar^{TM} StrepB to detect group B *Streptococcus*, CHROMagar^{TM} Salmonella Plus to identify *Salmolella*, CHROMagar^{TM} Listeria and CHROMagar^{TM} Vibrio.

2.6.2. Culture-independent method

In the recent years, culture-independent methods have gained increasing popularity, whether in safety monitoring or bacteria ecology study in the waters. Bacterial diversity study of river and coastal environments is essential to determine the ecology and evolution of bacteria to support management policies or to sustain risk assessment studies. The immense diversity of uncultured organisms has been revealed by using culture-independent methods. Thus, using culture-independent methods can provide the great window of information about diversity of the bacteria (Amann *et al*., 1995; Hugenholtz, 2002; Kemp and Aller, 2004; Alain and Querellou, 2009). At the end cultural independent methods are based on sophisticated gene technologies which

mostly have been used in medical research. These methods analyze whole genomic of selected genus such as 16SrRNA for microorganism. In last a few decades several different molecular techniques have been developed to monitor the microbial ecology and diversity in environment (Rastogi *at el*., 2011). There are wide range of cultural independent techniques such as PCR, Genetic fingerprinting techniques (such as REP-PCR, and DGGE), FISH and DNA microarrays that are used to study bacteria ecology (Rastogi *at el* , 2010; NHMRC, 2003). DNA microarray is used to test water samples for the actual genetic material of a microorganism rather than depend on microbial growth or using of microscope. Very large amount of DNA/RNA can be used on a small surface to detect microbes in sample by reaction with the complementary RNA or DNA form microbial population. This technique was developed by Stanford University and was named DNA microarray. It is expected that using this technique can reduce the time (to 4 hours) and cost of analyses for fecal indicators in environmental samples (NHMRC, 2003). FISH is another molecular technique which is used to detect microorganism. In this method fluorescent marker is attached to the DNA probe which is complementary to target of microorganism DNA that is being investigated. The sample can be subjected on a fixed surface generally microscopic slide is used. Thus, if the target microorganism present, it is resulting in glowing of target microorganism. This is then observed using a fluorescence microscope. A number of FISH methods have been developed to detect total coliforms and *Enterococci* (Fuchs *et al*., 1998; Meier *et al*., 1997; Patel *et al*., 1998). Denaturing Gradient Gel Electrophoresis (DGGE) is electrophoresis based method which use chemical gradient to denature the sample on acrylamid gel. This method has been used to analysis of the 16S rRNA genes from environmental samples. The result can be analyzed by obtained ban patterns (Muyzer et al., 1993). DGGE is rapid method many samples can be evaluated simultaneously. DGGE method is representing a powerful tool for monitoring microbial communities.

2.6.2.1 .Polymerase chain reaction (PCR)

The traditional method for detection of bacteria involves sampling and filtration, following by using other culturing techniques. This process is time consuming and the accuracy may not be high. Using the molecular technique shorten the time with high accuracy. One of the basic molecular techniques is polymerase chain reaction (PCR), which was developed by Kary Mullis in the 1980. PCR is based on using DNA polymerase ability to synthesize new strand of DNA which is complementary to the offered template. This technique is rapid, sensitive and specific for detection of indicator microorganism and pathogen. Several studies have been done to develop PCR techniques for rapid detection of *E. coli* and coliforms which confirm or detect the presumptive *E. coli* or coliforms within several hours (Fricker and Fricker, 1994). In general PCR is powerful technique to detect the pathogen because of its highly sensitive detection. It acts as an effective procedure to generate very large quantities of specific DNA sequence in vitro (Holland *et al*., 2000).

2.7. Bacterial Source Tracking

Each year, spend millions of dollars on monitoring of fecal coliform and *E. coli* in marine environment to determine water quality. However additional knowledge such as source of fecal contamination rather than just monitoring the bacteria level in water is required. Finding the source of contamination allows that desirable programs could be implemented to control the water quality and protect public and human health. Presence of fecal coliforms in water is considered as a threat for public health, because it shows the presence of bacterial pathogen (Simpson *et al*., 2002). Thus, finding the source of bacteria pollution helps in control and management of further pollution.

Bacteria source tracking (BST) can be carried out to identify the origin source of fecal pollution which can be come from human, pet, livestock or wildlife fecal bacteria in the water bodies (Scott *et al*.,, 2002; Simpson *et al*., 2002; Meays *et al*., 2004). There are several principles in BST methods. First, in contaminated water, there are multiple contributing sources of fecal pollution; each source has its own unique strains of bacteria. Molecular fingerprint is used as desirable method to determine the source of contamination. Second, a library of molecular fingerprints of bacteria is provided from the potential fecal bacteria in impaired water body. Third, molecular fingerprints of bacteria from contaminated water are compared with the known source fingerprints in the library (Dombek *et al*., 2000; Simpson *et al*., 2002)

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2.7.1. REP-PCR

Among different existing genotyping methods, repetitive extragenic palindromic elements-polymerase chain reaction (REP-PCR) contributes high taxonomic resolution and it may act as a rapid detector for evolution and diversity of the microbial genomic (Borges *et al*., 2003). The REP-PCR make use of DNA primer which is complementary to natural repeated, conserved DNA sequence within the genomics of bacteria, mostly in Gram-negative bacteria to provide strain specific fingerprints (Dombek *et al*., 2000). There are three different repetitive methods that have been employed in molecular methods, comprise repetitive extragenic palindromic (REP), enterobacterial repetitive extragenic consensus (ERIC) and BOX sequences (Versalovic *et al*., 1994; Borges *et al*., 2003). There is no specific primer for given microorganism and no information is required about interested genome. REP-PCR is desirable technique due product reproducibility and superior pattern and non-complex and quick result can be achieved (Carson *et al*., 2003).

When a large collection of sources is available to be compared, REP-PCR can be applied as valuable technique. However, this technique is limited to be used in small localities (McLellan *et al.*, 2003). REP-PCR has been proved more discriminatory than Restriction Fragment length Polymorphism (RFLP) and 16S rRNA PCR, and provides discriminatory power similar to randomly amplified polymorphic DNA (RAPD) (Kon *et al.*, 2009; Borges *et al.*, 2003). This technique has been found to be extremely reproducible, rapid, reliable and highly discriminatory (Versalovic *et al.*, 1994). REP-PCR has been successfully utilized to distinguish between different types of *E. coli* strains (Dombek *et al.*, 2000).

2.7.2. Other DNA based method in bacterial source tracking

There are many techniques have been used to determine the source of fecal contamination in environment. There is no "gold standard" method for bacteria source tracking. Currently the scientists are trying to use a combination of methods to detect the source of contamination (Scott *et al.*, 2002; USEPA 2005).

Several different molecular techniques have been applied in bacteria source tacking. The library based methods comprise Amplified Fragment Length Polymorphisms (AFLP) and Ribotyping and Plused-Field Gel Electerophoresis (PFGE). In AFLP method species specific adaptor and restriction enzyme are used (Vos, 1995). In PFGE methods the fragment variances in the whole genome can be observed. In Ribotyping the restriction enzyme is used to examine the fragment variances in the 16S ribosomal sequence. The library independent methods comprise host-specific PCR, based on length differences of host- specific genetic marker in genomic DNA or 16S rDNA of intestinal microorganism and t-RFLR which base on size of terminal end fragments (Simpson *et al.*, 2002; Meays *et al.*, 2004).

CHAPTER 3

METHODOLOGY

3. 1. Sampling

Water and sediment samples were collected in sterile bottles from 8 stations located along the Kuala Sepetang, Kuala Sangga Besar and Kuala Selinsing river of Kuala Sepetang mangrove estuary in Perak state, Malaysia. All samples were kept in ice box and transported to the laboratory in University of Malaya. Microbiological assays were performed within 72 h after sample collection.

The pH, salinity, temperature and depth of sampling site were measured in-situ with a multi-parameter liquid analyzer (model VSI -07). GPS reader was used to check the geographical coordinate

3.2. Bacterial enumeration and isolation

Water samples were shaken for 5 minutes. Different dilutions (10^{-1}, $10^{-2}$and 10^{-3}) of water samples in 0.85% of saline were provided and spread on duplicated CHROMagar™ orientation (CHROMagar Inc., Paris, France) and nutrient agar media. The plates were incubated at 37℃ for 24 h. On CHROMagar™ orientation the colonies developed in different color. The average number of each color of colony was determined by counting in duplicated plates. Three to four colonies were randomly picked from each varied colony and purified on CHROMagar™ orientation by streaking. The pure colonies sub-cultured on nutrient agar. The isolated bacteria were

then stabbed in nutrient agar and kept at room temperature. On nutrient agar the grown colonies were counted on each plate in different dilutions to check the total plate count.

One gram of each sediment sample was weighted and mixed into 9 ml of 0.85% of saline. The samples were shaken for 5 minutes and leaved for 1 minute. Three different dilutions (10^{-1}, $10^{-2}$and 10^{-3}) of each sediment sample in 0.85% of saline were prepared and spread on duplicated CHROMagar™ orientation and nutrient agar media. All the plates were incubated at 37℃ for 24 hs. The colonies grew in different color on CHROMagar™ orientation medium. The average number of each color of colony was determined by counting in duplicated plates. Three to four colonies were randomly picked from each varied colony and purified on CHROMagar™ orientation. The pure colonies sub-cultured on nutrient agar and stabbed in nutrient agar and kept at room temperature. On nutrient agar the grown colonies were counted on each plate in different dilutions to check the total plate count.

3.3. Bacterial identification

All isolated bacteria from CHROMagar™ orientation medium different in color (green, metallic blue, blue, brown, mauve, pink, colorless and purple) and morphology (rough, smooth, circle and oval) form water and sediment were revived on nutrient agar and streaked on CHROMagar™ orientation to check the purity of isolated bacteria. Pure cultures were then subjected to gram staining, oxidase and other biochemical tests for presumptive identification. Biolog Gen III MicroPlate™ test and specific PCR were also used to confirm the identity of bacterial isolates. CHROMagar Company claimed that different colors and colonies are produced by different type of bacteria as it summarized in Table 3.1 and Appendix 6.

Table 3.1. Typical Appearance of microorganisms on CHROMagar Orientation provided by CHROMagar manufacturer (http://www.chromagar.com).

Microorganism	Colony Appearance
E. coli	Dark pink to reddish
Enterococcus	Turquoise blue
Klebsiella, Enterobacter, Citrobacter	Metallic blue
Proteus	Brown halo
Pseudomonas	Cream, translucent
S. aureus	Golden, opaque, small
S. saprophyticus	Pink, opaque, small

3.3.1. Gram staining

The Gram staining test was used for each colony. One drop of sterile water placed into the surface of a clean glass slide, a single colony was picked and spread over a small area. Allow the film to air dry. The dried film was fixed by passing it briefly through the flame two or three times. The slide was flooded with crystal violet solution for up to one minute, the slide was washed off with tap water. Slide flooded with gram's Iodine solution up to one minute then washed. Then 95% alcohol was used to flood the slide for 10 to 15 seconds and washed off with tap water. Then at last part safranin solution was spread for 45 seconds and washed off with tap water .the slide was observed under microscope by applying mineral oil.

3.3.2. Gram Confirmation

A drop of 3% KOH was placed on a glass slide. A single colony was picked and placed on the slid and mixed with 3% KOH for 60 seconds. Those suspension gels became viscous and strings out when the loop was lifted (positive KOH reaction), the isolate is Gram negative. Those without any viscous and no strings out when loops was lift (negative KOH reaction), the isolate was Gram positive.

3.3.3. Oxidase Test

A small piece of filter paper was soaked in 1% kovács oxidase reagent and let dry. A sterile toothpick was used to pick a well isolated colony from a fresh (18- to 24-hour culture) culture plate and rubbed on the filter paper. The color change was observed. Microorganisms were oxidase positive when the color changed to dark purple within 5 to 10 seconds. Microorganisms were oxidase negative when the color did not change or it took longer than 2 minutes.

3.3.4. Catalase Test

Drop catalase test was applied in this experiment. A drop of 3% hydrogen peroxide was placed on a glass slide. A single fresh colony (18 to 24 hours culture) was picked and placed on the glass slide. Isolated bacteria were catalase positive which produced the bubbling. Microorganisms which did not produce bubbling were catalase negative.

3.3.5. Methyl Red Test (MR Test)

A loopful of fresh colony (18 to 24 hours culture) was inoculated into the test tube containing sterile MR-VP broth. The test tube was incubated at 37˚C for 24 to 48 hours. 1ml of the inoculated MR-VP broth was aliquoted into another sterile test tube. The remaining broth was added with a few drops of Methyl Red reagent and observed the color change immediately. Microorganisms were MR positive when red color was appeared. Microorganisms with no color change were MR negative.

3.3.6. Voges Proskauer Test (VP Test)

The aliquated 1ml of the inoculated MR-VP broth was then added with alpha-naphthol and 40% potassium hydroxide in 3:1 ratio (i.e. 300µl:100 µl). The tube was allowed to stand for 20 to 30 minutes for color development to occur. Those culture turn to red to pink color were VP positive and those microorganisms appeared yellowish to copper in color were VP negative.

3.3.7. Simmon's Citrate Test

Fresh (18- to 124-hour culture) pure culture was used as an inoculation source. A single well isolated colony was picked and lightly streaked on the surface of citrate agar slant. The tube was incubated with a loose cap at 37˚C for 24 hours. Simmon citrate positive isolated colonies were identified by appearing blue color. Microorganisms with negative citrate reminded in green color constant green.

3.3.8. Indole Test

SIM medium was used to check the indole test in this experiment. A pure fresh (18-24 hours) culture was used as an inoculation source. A single isolated colony was stabbed in sterile SIM medium. The inoculated tube was incubated at 37˚C for 24 hours. Five to ten drops of kovac's reagent were added to target tube. Positive indole bacteria were detected by producing the red ring at the top of the tube. The negative indole bacteria did not produce any red ring at the top of the tube.

3.3.9. Motility Test

SIM medium was used to check the motility test in this experiment. A Fresh (18-24 hours) culture was stabbed into SIM medium. The inoculated tube was incubated at 37˚C for 24 hours. The motile bacteria were identified by producing the cloudy medium and the less distinct line of growth. Non motile bacteria produced the clear visible growth line.

3.3.10. Morphology on EMB Medium

EMB medium was used as selective medium to isolate and detect *Enterobacteriaceae* family. The pure fresh (18 to 24 hours) culture colonies were streaked on EMB medium. Plates were then incubated at 37˚C for 24 hours. Developed colonies were identified based on their color and morphology on EMB.

3.3.11. Morphology on MacConkey Medium

MacConkey medium was used to identify the *Enterobacteriaceae* family. Pure cultures were streaked on this medium. Streaked plates were then incubated at 37˚C for 24 hours. The gown colonies were detected based on their color and morphology on macconky.

3.3.12. Morphology on CHROMagar^{TM}ECC Medium

This medium was used to identify *E. coli*. The fresh (18 to 24 hours) culture colony was sub-cultured on CHROMaga^{TM} ECC medium. The streaked plate was incubated at 37˚C for 24 hours. *E. coli* strains indicated as blue to green color on this medium.

3.4. Biolog GeneIII Microplate Test

Isolated bacteria identifying by biochemical test were streaked on nutrient agar. Streaked plates were then incubated at 37˚C for 16 to 24 hours. Culture should be used freshly. Recommended incubation period for most bacteria is around 4 to 24 hours. Those spore forming Gram-positive bacteria (Bacillus and related genera) should be grown for less than 16 hours to minimize sporulation**.**

Turbidity meter was used to obtain the desirable turbidity which was 95% to 98%. Clean tube containing uninoculated IF was used for blanking the turbidity meter. The transmittance was set at 100s, so that the meter reads 100%. Blanking process was repeating for each sample.

A cotton-tipped inoculatorz swab was applied to pick up colony from a pure culture cell. The swab was then stirred inside IF solution to obtain a uniform cell

suspension. The turbidity of tube was read by the turbidity meter. The cell suspension was poured into the multichannel pipet reservoir. All the wells were filled dpipettor tips were then ejected. All the MicroPlates were incubated at 33˚C for 18 to 24 hours.

All the MicroPlates were read using Biolog's Microbial Identification Systems software (e.g.OmniLog® Data Collection).

3.5. Recovery of *Enterobacteriaceae* on CHROMagar orientation using enrichment and pre-enrichment

Water samples (10 gram sediment added to 100 ml 0.85% saline) were shaken for 5 minutes. Samples were passed though cloth mesh and paper filter to remove the clay and sand. 50 ml of each sample was concentrated onto a sterile 47-mm diameter membrane (pore size, 0.45±0.02 µm). A sterile forceps used to directly transfer the membranes into10ml sterile BPW (Buffer Peptone Water) and incubated at 37˚C for 4-6 hours as pre enrichment and 24 hours as enrichment process. BPW culture broths were then sub-cultured onto CHROMagar™ orientation. The presumptive *enterobacteriaceae* were isolated and analyzed.

3.6. *Escherichia coli* identification and confirmation with polymerase chain reaction (PCR)

3.6.1. DNA Template preparation

Pure isolated bacteria were sub-cultured on Luria Bertani Agar plates to extract the crude DNA using boiling cell extraction method. A pure single colony of bacteria was inoculated in 100µl of sterile Luria Bertani broth in 1.5ml eppendorf tube and incubated at 37˚C overnight. Cultured tube was centrifuged at 13,400 rpm for 3 minutes. The supernatant was discarded. Approximately 600µl of sterile Phosphate buffered saline (PBS) was added to cell pellet and re-suspended. The cell suspension was then re-centrifuged at 13,400 rpm for 3 minutes. Supernatant was removed and 600µl 1x TE was added and suspended. The tube was centrifuged at 13,400 rpm for 3 minutes. After discarding the, 100µl of 1x TE was added and suspension was then heated at 99℃ for 10 minutes, the tube was then chilled on ice for 20 minutes. An aliquot of 80 µl supernatant was transferred to a new sterile microfuge tube for further analysis. A 5 µl of this crude DNA was used as template for PCR.

3.6.2. PCR reactions

Presumptive *E. coli* isolates based biochemical tests were further subjected to PCR assay targeting the *phoA* gene, which is the housekeeping gene for *E. coli* (Kong *et al*., 1999). This monoplex PCR assay was carried out in total volume of 25 µL. The master mixtures were prepared as listed in Table 1.1 (Appendix 1). PCR reaction was carried out using the following cycling conditions in Table 3.3. PCR products were analysed by separating on the 1.5% of agarose gel, and visualised under UV after staining with Gel red nuclide acid stain. The primer sequence for detection of the *phoA* in *E. coli* housekeeping is in Table. 3.2.

Before starting the PCR assay desirable preparation was required for a coordinated work flow. First, PCR reagents such as 5 X PCR Buffer (GoTaq® Flexi Buffer), dNTPs and $MgCl_2$ stock were thawed on ice. They were spun briefly before using. In order to prolong shelf time of the PCR materials, they were prepared in batches and aliquot for storage to prevent contamination of stock solutions.

For the preparation of master mixtures, the DNA template was thawed on ice and centrifuged briefly. Primer, 5x PCR Buffer, $MgCl_2$ and dNTPs were thawed on ice and spun briefly before being used. The number of PCR tubes needed was estimated and they were labelled accordingly.

PCR biosafety cabinet was cleaned using 70% ethanol before the preparation starts. The 0.2 ml, 1.5 ml eppendorf tubes, ddH_2O in 1.5ml tubes, pipette tips, micropipettes and PCR tube rack were exposed under UV in the PCR cabinet for 15 min before starting the work. Gloved hands were cleaned with ethanol as well. After that, ddH_2O, 5x PCR Buffer, dNTPs, $MgCl_2$, primer were added into 1.5ml tube accordingly. *Taq* Polymerase was added into a 1.5 ml tube al the last. The master mixture was then spun briefly before dispensing into 0.2 PCR tubes. Lastly, 5 µL of DNA template was added into the 0.2 ml PCR tube. Each reaction included a negative control, which was a reaction mixture which contained water instead of DNA template, and positive controls that included purified extracted DNA from a known strain of *E. coli.* Finally, before loading the 0.2 ml PCR tubes in the Thermal Cycler, all the tubes were spun briefly. The PCR was run under the appropriate program as in Table 3.3. The PCR products were then analyzed using 1.5% agarose gel.

Table 3.2. Primer used to detect *E. coli* housekeeping gene in monoplex PCR assay (Kong *et al*., 1999).

Target gene	Primers	Sequences	Expected band
Alkaline phosphatase *(phoA)*	*Pho-F*	GTC ACA AAA GCC CGG ACA CCA TAA ATG CCT	903bp
	Pho-R	TAC ACT GTC ATT ACG TTG CGG ATT TGG CGT	

Table 3.3. Condition of monoplex PCR assay targeting *phoA* gene of *E. coli*

Condition	Temperature(℃)	Duration(min)	Cycles
Pre-denaturation	94	2	1
Denaturation	94	1	35
Annealing	56	1	
Extension	72	1	
Final extension	68	1	1
Hold	4		

3.6.3. PCR amplification of 16S rDNA fragments

For reconfirmation of identified bacteria by Biolog GenIII (such as Enterococci, Klebsiella and Enterobacter) 16S rDNA was amplified from the isolated genomic DNA by using a pair of universal primers f, (5-CCT ACG GGA GGC AGC AG-3) and r (5-CCG TCA ATT CMT TTG AGT TT-3)…. was performed in a total volume of 25 µl. The master mixtures were prepared as listed in Table 1.3 (Appendix 1). PCR reaction was carried out using the following cycling conditions in Table 3.4. The PCR products were load on the 1.5% of agarose gel and visualized under UV after staining with Gel red nucleic acid stain. PCR amplicons were purified and sent for sequencing.

Table 3.4. Condition of PCR assay targeting 16S rDNA gene

Condition	Temperature(℃)	Duration(min)	Cycles
Pre-denaturation	95	5	1
Denaturation	95	1	35
Annealing	55	1	
Extension	72	1	
Final extension	72	5	1
Hold	4		

3.6.4. PCR amplicon purification

One hundred µl of each reaction product was transferred in to a 1.5 microcentrifuge tube. 5 volumes of DF buffer was added to one volume of the sample and mixed by vortex. A 1.5 microcentrifuge tube (DF tube) was placed into 2ml tube (collection tube). The sample mixture which was mixed by vortex was transferred to DF column and centrifuged at 13.400x g for 30 seconds. Then the flow-through was discarded and DF column was placed back in the 2 ml collection tube.

In washing step, 600µl of wash buffer was added into the center of the DF column and let stand for one minute. Then the DF column was centrifuged for 30 seconds at 13.400 x g. The flow- through was discarded and DF column was placed back in the 2 ml collection tube. The DF column was then centrifuged again at 13.400 x g for 3 minutes to dry the column matrix. Died DF column was transferred to a new 1.5 microcentrifuge tube. 50µl of elution buffer was added to the center of the column matrix. The tube was stood for 2 minutes until the elution buffer was completely absorbed by the matrix. The tube was centrifuged for 2 minutes at 13.400 x g to elute the purified DNA. Then the purified DNA was sent for sequencing by 1stBASE Sdn. Bhd. (Selangor, Malaysia).

3.7. Comparison of coliforms and *E. coli* identification accuracy of CHROMagar™ Orientation, CHROMagar™ ECC, EMB and MacConkey medium

The enrichment was carried in this comparison. Water samples were shaken for 5 minutes. Samples were passed though cloth mesh and paper filter to remove the clay and sand. 50 ml of each sample was concentrated onto a sterile 47-mm diameter membrane (pore size, 0.45±0.02 µm). A sterile forceps used to directly transfer the

membranes into10ml sterile BPW (Buffer Peptone Water) and incubated at 37°C for 24 h. BPW culture broths were then sub-cultured onto CHROMagar^{TM} orientation, CHROMagar^{TM} ECC and EMB media to compare these media for isolation of *E. coli.* CHROMagar^{TM} orientation, EMB and MacConkey were used to isolate *Klebsiella* sp and *Enterobacter* sp. All plates were incubated at 37 °C for 24 h. Three to four presumptive *E. coli,* and coliforms *(Klebsiella* sp. and *Enterobacter* sp.*)* colonies were picked from each different plate and purified on the same medium. The pure colonies were streaked on nutrient agar. The isolated bacteria were stabbed in nutrient agar and kept at room temperature for further assaying.

3.8. Repetitive extragenic palindromes (REP)-PCR amplification

REP-PCR was carried out to amplify repetitive elements from bacterial isolates to determine the genetic diversity of these isolates. The REP-PCR was used following method described by Lim *et al*. (2009). REP-PCR was performed in a total volume of 25 µl. The master mixtures were prepared as listed in Table 1.2 (Appendix 1). PCR reaction was carried out using the following cycling conditions in Table 3.5. The PCR products were analysed on the 1.5% of agarose gel, and visualised under UV after staining with Gel red nucleic acid stain. The sequence of the oligonucleotide primer used for REP-PCR assay was GCG CCG ICA TGC GGC ATT (Lim *et al*., 2009).

Table 3.5. REP- PCR Condition

Condition	Temperature(℃)	Duration	Cycles
Pre-denaturation	94	7mins	1
Denaturation	94	30 sec	
Annealing	56	1 min	30
Extension	72	8 min	
Final extension	68	16 min	1
Hold	4		

3.9. Data analysis

Data were analyzed by using Statistical Package for the Social Sciences (SPSS) was used to analyze tables in this study. BioNumerics software (Applied Maths, Beligium) was used to construct dendrograms using the uweighted pair group method with arithmetic means (UPGMA) tree building method.

CHAPTER 4

RESULT

4.1. Samples collection and physicochemical parameters

Samples Collections for this study was done on July 2011 and February 2012 in Kuala Sepetang, Malaysia. Water and sediment samples were collected from eight stations from upstream to downstream along the salinity gradient of the estuary. The locations of sampling stations are showed in Fig 4.1. The GPS coordinates and physicochemical parameters measured at each stations are indicated in Table 4.1 and 4.2 .

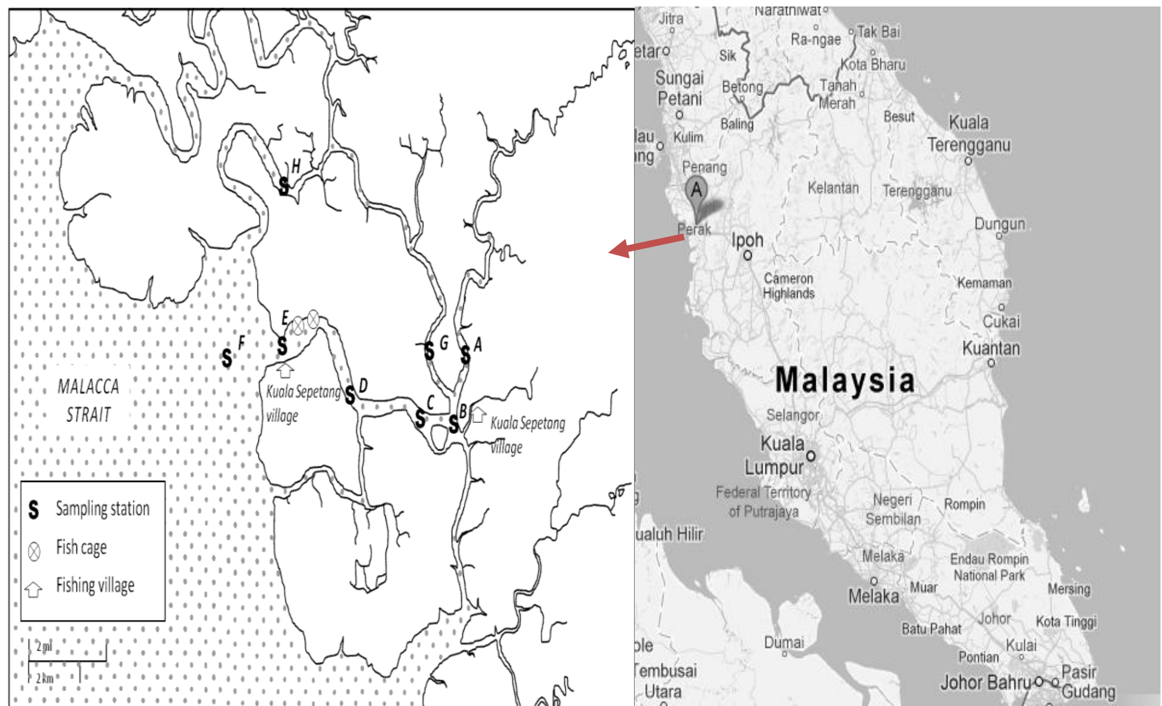


Fig 4.1. Locations of sampling sites along Kuala Sepetang estuary in the study.

Table 4.1. GPS coordinates and physicochemical parameters measured at each station during the study in 2011

Station	GPS reading (N)	GPS reading (E)	Time	Temperature, ºC	pH	Salinity	Depth (m)
A	4.85167	100.627	9:08	30.03	8.96	20.66	5.5
B	4.83902	100.6231	12:28	31.25	9.91	20.27	2.5
C	4.83981	100.6151	10:01	30.52	9.84	25.77	5.9
D	4.84335	100.5868	10:21	30.3	9.84	25.9	1.7
E	4.85813	100.5602	11:28	30.39	9.79	26.93	2.3
F	4.85162	100.5361	11:16	30.35	9.81	27.09	2.4
G	4.84763	100.6176	9:30	29.3	9.28	20.89	3.5

Table 4.2. GPS coordinates and physicochemical parameters measured at each station during the study in 2012

Station	GPS reading (N)	GPS reading (E)	Time	Temperature, ºC	pH	Salinity	Depth (m)
A	4.85167	100.627	9:50	29.89	6.39	10.34	5.5
B	4.83902	100.6231	11:25	30.18	6.85	11.11	2.5
C	4.83981	100.6151	12:05	30.14	6.85	14.18	5.9
D	4.84335	100.5868	12:30	30.6	7.31	16.1	1.7
E	4.85813	100.5602	13:10	31.47	7.4	17.34	2.3
F	4.85162	100.5361	13:45	31.14	7.76	18.03	2.4
G	4.84763	100.6176	11:14	31.1	7.01	12.04	3.5
H	4.87872	100.6048	1:12	30.14	6.95	15.7	12.5

4.2. Total viable cell count on Nutrient agar and CHROMagar^{TM} Orientation

In this study, the water and sediment samples were enumerated for total viable microbial cells on both, CHROMagar^{TM} Orientation and Nutrient agar with direct-plating approach. All colonies formed on nutrient agar and CHROMagar^{TM} Orientation after overnight incubating at 37°C were enumerated. The result showed that in both nutrient agar and CHROMagar^{TM} Orientation medium, the population of viable cell count in sediment was higher than in water from all stations (Table 4.3). However, Nutrient agar yielded higher total viable cell count in the sediment and water samples than CHROMagar^{TM} Orientation (Table 4.3). Nonetheless, CHROMagar^{TM} Orientation yielded not just total viable cell count, but differentiation of cells of distinct color and morphology (Fig4.2). A higher viable cell counts was observed at stations A, B and D which were close to the human settlement.

Direct plating of sediment and water samples on CHROMagar^{TM} Orientation always yielded lesser counts than nutrient agar. *Enterobacteriaceae* was accounted at lower rate on CHROMagar^{TM} Orientation with direct plating. To increase the recovery rate of *Enterobacteriaceae* on CHROMagar^{TM} Orientation, pre-enrichment-plating approach was tested. With an extra step of pre-enrichment, a higher enumeration rate of total viable count was observed (Fig4.2). The isolated bacteria with pre-enrichment-plating approach were predominantly *Enterobacteriaceae.*

Table 4.3. Comparison of cultivable bacteria count in waters and sediments between CHROMagar^{TM} Orientation and Nutrient agar using direct plating

Station	Cultivable bacteria count (log10 cfu/g or 100ml):			
	CHROMagar^{TM} Orientatio		**Nutrient agar**	
	water	**Sediment**	**Water**	**Sediment**
A	5.6±0.7	6.2±0.8	6.7±0.2	6.6±0.6
B	5.6±0.3	6.2±0.3	6.5±0.2	6.5±0.2
C	5.4±0.2	5.8±1.6	5.7±0.5	6.9±0.2
D	5.1±0.4	5.6±1.9	6.1±0.4	7±0.4
E	4.6±0.8	5.7±1	5.2±0.5	6.9±0.5
F	4.5±1.7	6±1.2	6±0.2	7.1±0.1
G	5.6 ±0.2	6.2± 0.7	5.6± 0.1	6.6± 0.1
TOTAL	**5.2±0.5**	**5.9±0.3**	**6±0.5**	**6.8±0.2**

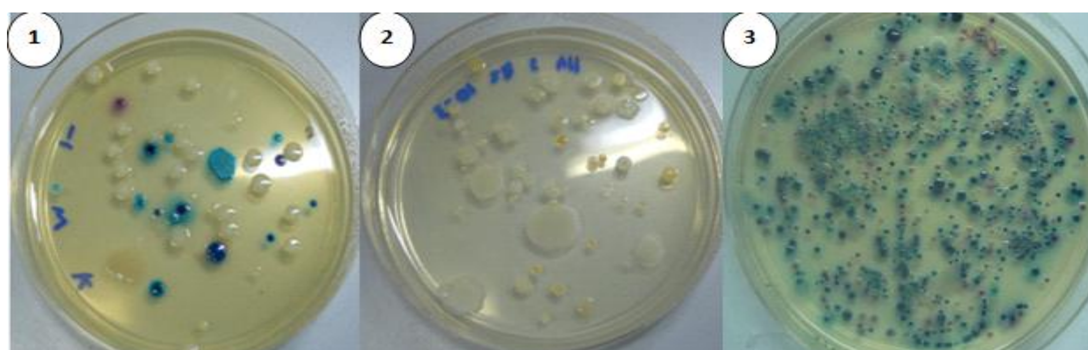


Fig 4.2 Total viable cell count on Nutrient Agar (2, direct plating) and CHROMagar^{TM} Orientation (1, direct plating. 3, pre-enrichment) after overnight incubation at 37° C.

4.3. Culturable estuarine bacterial population on CHROMagar^{TM} Orientation

Direct culturing of estuarine water and sediment samples collected along a salinity gradient in Sangga Besar River on CHROMagar^{TM} Orientation resulted in isolation and enumeration of 21 distinguishable colony morphotypes. These morphotypes were characterized by their distinctive colony colors, presence of pigment diffusion around the colony and other common morphologies on CHROMagar^{TM} Orientation.

A great spectrum of colony colors, ranged from colorless to green, blue-green, green-blue, blue, violet-blue, blue-violet, violet, red-violet and violet-red were defined. As color definition can be quite subjective from person to person, identification and defining of colony color on CHROMagar^{TM} Orientation was performed with reference to the Garderner's Color Wheel (The Color Wheel Company, Philomath, US) for a standardized and more accurate color definition (Appendix 4).

For simplicity and ease in colony counting of a great numbers of plate, the morphotypes were grossly categorised as purple (violet, red-violet and violet-red), blue (blue, violet-blue and blue-violet), green (green, blue-green and green-blue) and colorless morphotype (Fig. 4.4). The result showed that different morphotypes were enumerated in both water and sediment samples along the salinity and pH gradient in Kuala Sepetang estuary in 2011 and 2012 (Fig.4.3 and Fig 4.4). The result showed that the changing of salinity and pH was different in both 2011 and 2012. The highest level of pH and salinity was observed in 2011. The changing in salinity and pH effected on the predominate morphotypes in each of the station. It was observed that the colorless and green morphotypes were predominated in both the water and sediment samples.

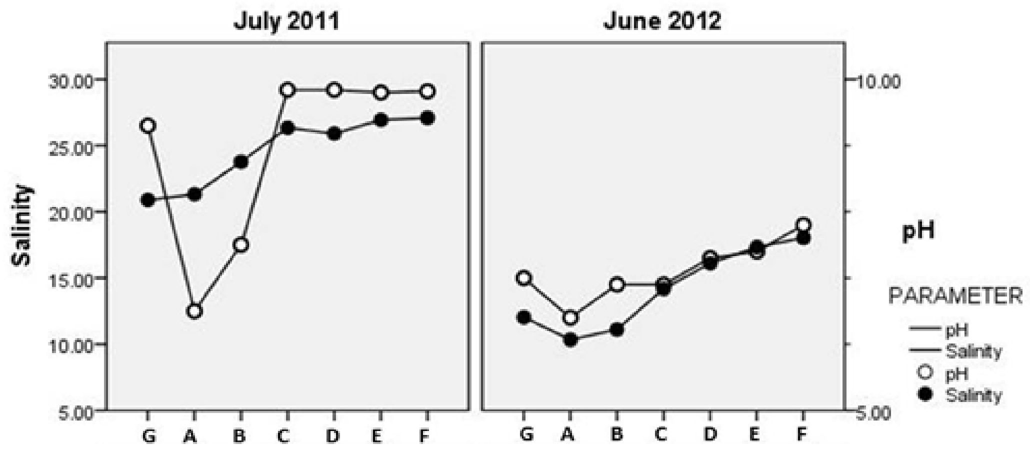


Fig.4.3. Change of water salinity and pH in seven different locations at July. 2011 and Jun. 2012

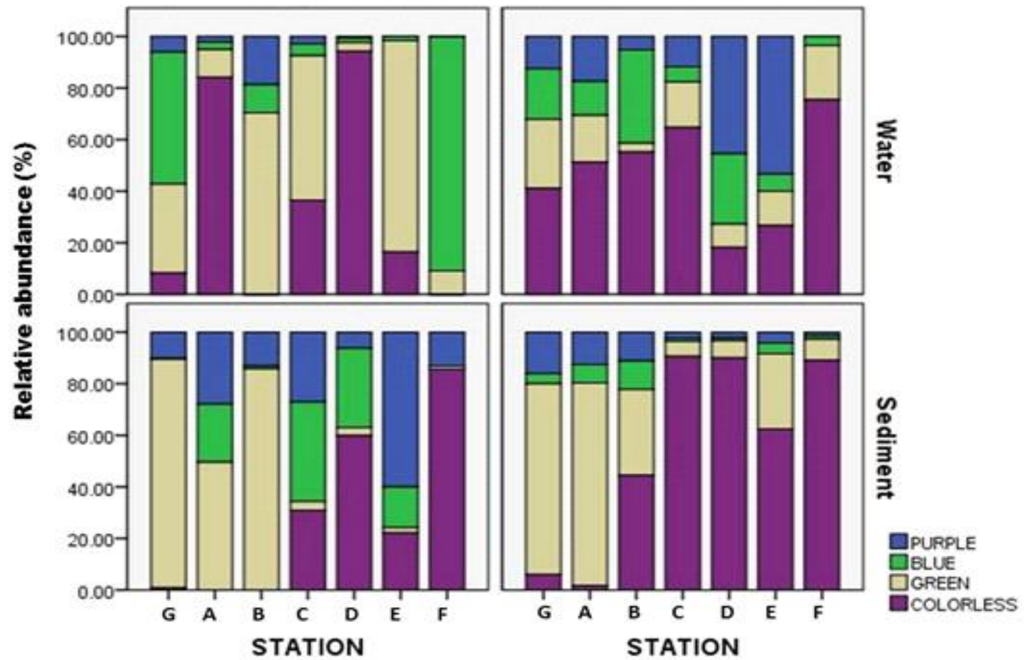


Fig 4.4. The cultivable bacteria population at seven different locations in estuarine waters and sediments on CHROMagar™ Orientation

4.4 Isolation and identification of fecal indicator bacteria and potential human pathogens from estuarine waters and sediments using CHROMagar^{TM} Orientation

A total of 520 isolated bacteria of different in colony color and morphology were collected from the water and sediment samples (Fig. 4.5 and 4.6). All isolates were subjected to identification with biochemical assays (e.g. Gram staining and microscopy, oxidase test, catalase test, etc.), Biolog Gen III microplate test (Fig. 4.7) and specific PCR (only for presumptive *E. coli* isolate).

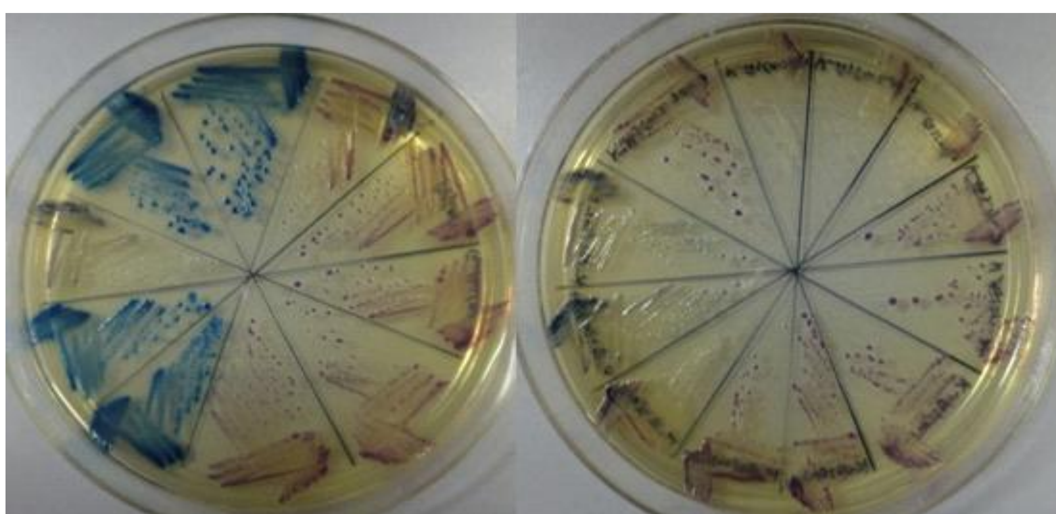


Fig 4.5. Reviving the isolated bacteria on CHROMagar^{TM} Orientation

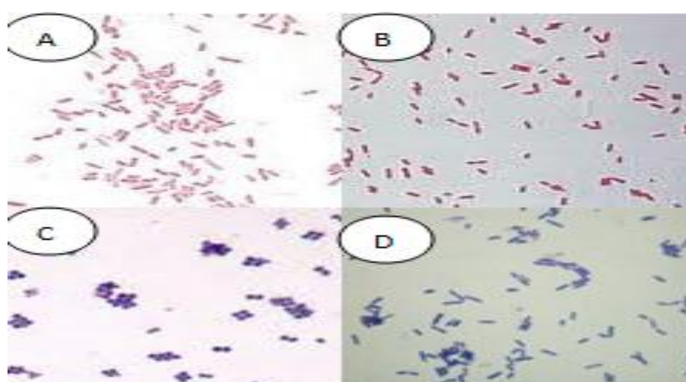
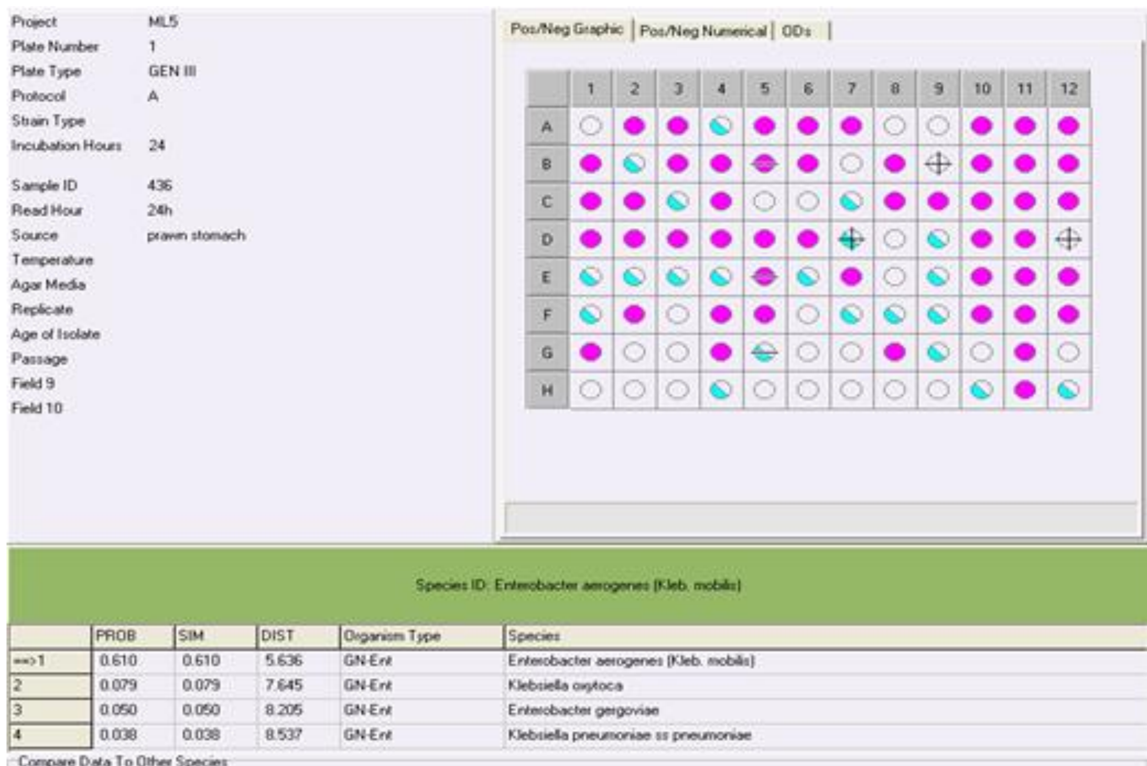


Fig 4.6. x1000 Gram-staining test was carried out to all isolates; A, *E. coli*; B, *Klebsiella*; C, *Staphylococcus*; D, *Bacillus*



4.7. Representative biochemical profile using Biolog Gen III to identify *Enterobacter aerogenes*

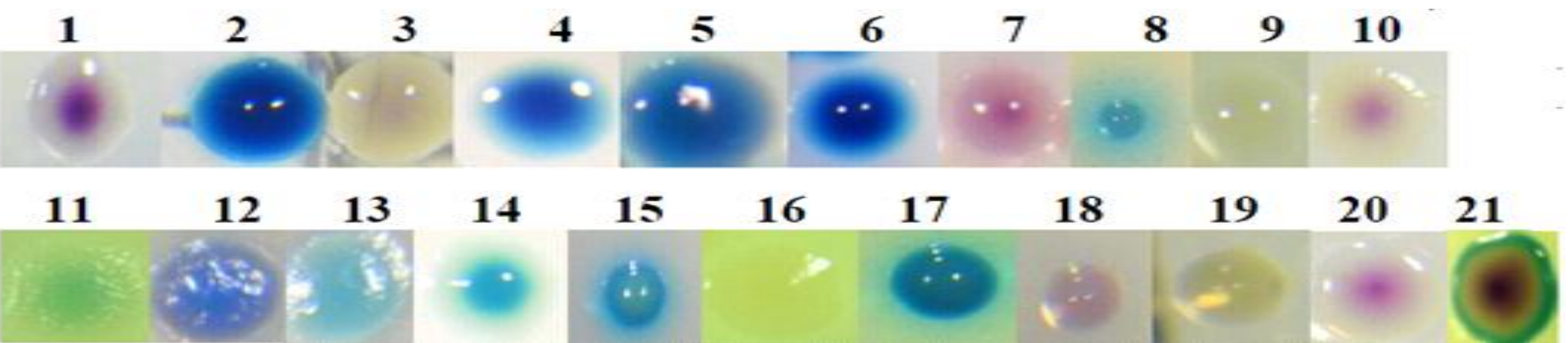
Out of 520 isolated colonies, 117 colonies were Gram-positive and 403 colonies were found as Gram negative. The result showed (Table 4.4). Noteworthy, 403 isolates (76.9%) were Gram negative rod-shaped bacteria; 33 isolates (6.3%) were Gram positive *cocci;* and 84 isolates (16.0%) were Gram positive bacilli. These isolates comprises of 17 bacterial genuses that encompassed 31 species.

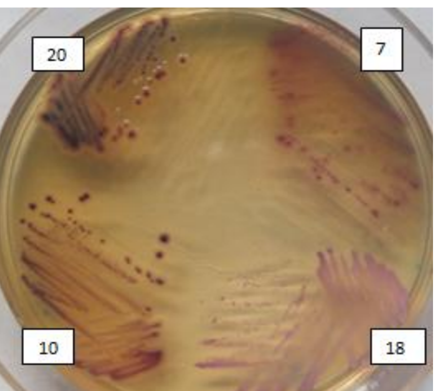
Approximately three quarters of the isolated Gram negative bacteria were *Enterobacteriaceae*. These 520 estuarine isolates comprised of 28.4% of coliforms (*Citrobacter* spp., *Enterobacter* spp., *Hafnia* spp.*, Klebsiella* spp.*, Serratia* spp. and *Raoutella* spp; 28.6% of *Escherichia coli* (*E. coli);* 4.2% of *Enterococci;* and 38.0% of other bacteria (Table 4.4). In another words, more than half (61.2%) of the bacteria isolated in this study were one of the three commonly used fecal indicators in microbiological monitoring of waters and marine environments.

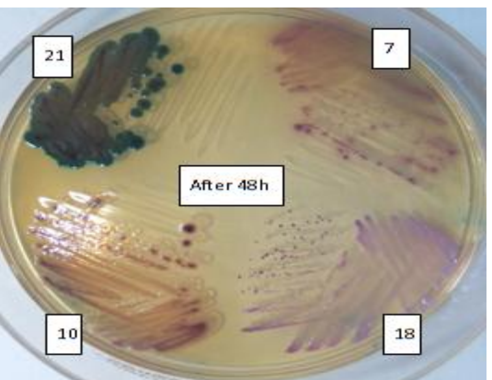
All identified bacteria could differentiate by their colony characteristics which are described in Table 4.4 and indicated in Fig. 4.8. *Bacillus* sp, *Hafnia alvei, Exigubacteria*, *Serratia marcescens, Escherichia coli* and *Citrobacter koseri* colonies on CHROMagar^{TM} Orientation could be easily differentiated. Although *Entrobacter aerogenes, Klebsiella oxytoca* and *Raoutella planticola/orthinolytica* produced the similar colony color, they were differentiated by their edge color (Table 4.4).

There was similarity in pigment production in *Vibrio* and *Aeromonas* isolates. The differentiation was identified by the appearance of green edge color in *Vibrio* colonies after 24 hours of incubation.

Staphylococci were clearly intelligible: *S.hominis hominis* and *S. saprophyticus* produced red-violet to pink entire colonies. *S. sciuris rodentium* demonstrated green-blue color which was similar to *Enterococcus* and *Paenibacillus popillae* colonies. *Enterococcus* and *Paenibacillus papillae* were distinguishable by their aqua blue diffusion and greenish blue edge, respectively







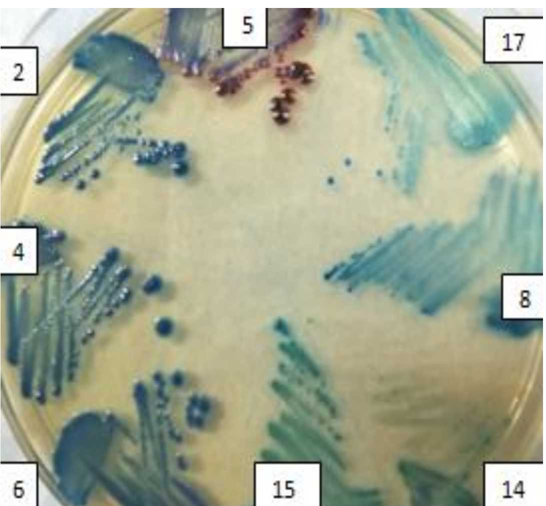


Fig 4.8. Colony color of selected bacteria CHROMagar^{TM} Orientation 1,*C. Koseri*; 2,*E. aerogenes*; 3,*H.alive*; 4,*K. Oxytoca*; 5, *S. marcescens*; 6,*R. Planticola*; 7,*E. coli*; 8,*Entrococcus casseliflavus*; 9,*Acinetobactor johansonii*; 10,*Aeromonas sp*; 11,12,13,*Bacillis sp*; 14,*Exigubacteria sp*; 15,*Paenibacillus papillae*; 16,*Pesudomonase sp*, 17,*Staphylococcus sciuris*; 18,S. *hominis ss hominis* and *S.saprophyticus*; 19, *Stenotrophomonas rhizophila*; 20,*Vibrio sp*; 21, *Vibrio sp* at room temperature.

Table 4.4. Pigment reaction and colony morphology of Gram-negative and -positive bacteria on CHROMagar ™ Orientation

Fecal indicators	Genus	Species	Colony morphology:									No. of isolates	%
			Colony color	**Edge color**	**Diffused-halo**	**Form**	**Elevation**	**Margin**	**Surface**	**Structure**	**Colony size (mm)**		
Coliform	Citrobacter	*Citrobacter koseri/youngae* *Citrobacter* spp.	Red-violet	white to pale yellow	-	Circular	Low convex	Entire	Smooth	Translucent	0.5-1.5	2	0.4
	Enterobacter	*Enterobacter aerogenes*	Blue	-	-	Circular	Low convex	Entire	Smooth	Translucent	1.0-1.5	20	3.8
		enterobacter clocae dissolvens	Blue	-	-	Circular	Low convex	Entire	Smooth	Opaque	1		
	Hafnia	*hafnia alvei*	White (with sheer Red-violet pigmentation)	-	-	Circular	Low convex	Entire	Smooth	Opaque	1	1	0.2
	Klebsiella	*Klebsiella oxytoca*	Blue-violet	Colorless	-	Circular	Low convex	Entire	Smooth	Opaque	2	110	21.2
	Serratia	*Serratia marcescens. Marcescen*	Blue-Violet	Red-violet	-	Circular	Low convex	Entire	Smooth	Opaque	1.0-1.5	4	0.8
	Raoutella	*raoultella planticola/ orthinolytica*	Blue-violet	Colorless	-	Circular	Low convex	Entire	Smooth	Opaque	2	12	2.3
Facal coliform	Escherichia	*E. coli*	Violet-red	Pale violet-red	Violet-red	Circular	Low convex	Entire	Smooth	Translucent	0.5-1.5	150	28.8
Fecal indicator bacteria	Enterococcus	*enterococcus casseliflavus*	Green-blue	-	Green-blue	Punctiform	Dome-shape	Entire	Smooth	Translucent	0.2	22	4.2
non-fecal indicator	Acinetobacter	*Acinetobacter johansonii*	Colorless (milky in color)	-	-	Circular	Low convex	Entire	Smooth	Opaque	1.0-1.5	1	0.2
	Aeromonas	*Aeromonas allosacchallophila* *Aeromonas encheleia* *Aeromonas media-like DNA group 5*	Red-violet	Colorless/ Pale yellow	-	Circular	Umbonate	Entire	Smooth	Translucent	1.5-2.5	46	8.8

	Aeromonase bestiarum											
	Aeromonase veronii DNA group 10											
Bacillus	*Bacillus megaterium* *Bacillus slarius*	Green/ Blue-green/ Green-blue/ Blue/ Red-violet	-	-	Circular/ irregular	raised/ Low convex	Entire/ undulate	Waxy/ rough	Opaque	1.0-3.0	71	13.7
	Bacillus cereus/psedomycoides *Bacillus cereus/thuringiensis* *Bacillus pumilus*											
Exiguobacteria	*Exiguobacterium aurantiacum* *Exiguobacterium acetylicum*	Green/ Blue-green	White/ pale yellow	-	Circular	Low convex	Undulate	Smooth	Translucent	0.5-1.0	8	1.5
Paenibacillus	*Paenibacillus popillae*	Blue-green (with green sheen)	-	Blue-green	Circular	Dome-shape	Entire	Smooth	Opaque	0.5-0.8	5	1.0
Pseudomonas	*Pseudomonas putida biotype B*	Colorless	-	-	Circular	Low convex	Entire	Smooth	Translucent	2	6	1.2
Staphylococcus	*Staphylococcus sciuris ss rodentium*	Green-blue	-	Green-blue	Circular	Dome-shape	Entire	Smooth	Opaque	0.5	11	2.1
	Staphylococcus hominis ss hominis	Red-violet	-	-	Circular	Dome-shape	Entire	Smooth	Opaque	0.5		
	Staphylococcus saprophyticus	Red-violet	-	-	Circular	Dome-shape	Entire	Smooth	Opaque			
Stenotrophomonas	*Stenotrophomonas rhizophila*	Green (deepened to blue-green after 48 hour)	Transparent	-	Irregular	Low convex	Undulate	Smooth	Translucent	0.5-1.0	24	4.6
Vibrio	*Vibrio cholerae 01/0139* *Vibrio fluvialis*	Violet-red	Transparent (turn into blue-green over prolong incubation)	-	Circular	Low convex	Entire	Smooth	Translucent	1.0-3.0	27	5.2
										TOTAL	**520**	**100**

4.5. Comparison of the performance of CHROMagar^{TM} Orientation, CHROMagar^{TM} ECC, EMB and MacConkey agar for accurate detection of fecal indicators in estuarine samples

The comparison between CHROMagar^{TM} Orientation, CHROMagar^{TM} ECC and EMB was summarized in Table (4.5). The accuracy of CHROMagar^{TM} Orientation was evaluated for presumptive detection of *E. coli* in comparison with CHROMagar^{TM} ECC and EMB. Presumptive *E. coli* strains (n=42), were picked by its specific color and colony characteristics on each medium and identified using biochemical and Biolog GENIII Microplate system. The result showed (Table 4.5) that the accuracy of EMB to detect *E. coli* was 88.1%. 37 presumptive *E. coli* strains were confirmed as *E. coli*. 40 presumptive strains were confirmed as *E. coli* strains on CHROMagar^{TM} ECC, showing the accuracy of 95.2% to detect *E. coli* strains which was the highest accuracy compared to CHROMagar^{TM} Orientation and EMB. CHROMagar^{TM} Orientation showed 92.9% of accuracy in identification of *E. coli*. 39 out of 42 pink color colonies with pink diffusion were confirmed as *E. coli* strains on CHROMagar^{TM} Orientation.

CHROMagar^{TM} Orientation was evaluated for detection of coliform bacteria (*Klebiella* sp and *Entrobacter* sp) (Table 4.6) from estuarine water samples. 41 presumptive coliform colonies were picked from each different media (CHROMagar^{TM} Orientation, MacConkey and EMB). The result showed that the accuracy of this medium was 95.1% in detection of coliforms (*Klebiella* spp and *Entrobacter* spp) which was the higher accuracy in compare to MacConkey and EMB with the accuracy of 87.8% and 92.7%, respectively.

Table 4.5. Accuracy of CHROMagar^{TM} Orientation, CHROMagar^{TM} ECC and EMB for presumptive identification of *E. coli*

Estuarine samples	No. of isolates tested	Media tested:		
		Eosin Methelene Blue agar	CHROMagar^{TM} ECC	CHROMagar^{TM} Orientation
A	7	6 (85.7)	6 (85.7)	7 (100.0)
B	7	7 (100.0)	6 (85.7)	6 (85.7)
D	7	7 (100.0)	7 (100.0)	6 (85.7)
E	7	6 (85.7)	7 (100.0)	6 (85.7)
G	7	6 (85.7)	7 (100.0)	7 (100.0)
H	7	5 (71.4)	7 (100.0)	7 (100.0)
TOTAL	**42**	**37 (88.1)**	**40 (95.2)**	**39 (92.9)**

Table 4.6. Accuracy of CHROMagar^{TM} Orientation , MacConkey and EMB for presumptive identification of coliform

Estuarine samples	No. of isolates tested	Media tested:		
		Eosin Methelene Blue agar	MacConkey	CHROMagar^{TM} Orientation
A	6	6 (100.0)	4 (66.7)	6 (100.0)
B	6	6 (100.0)	5 (83.3)	5 (83.3)
D	5	5 (100.0)	4 (80.0)	5 (100.0)
E	6	6 (100.0)	6 (100.0)	6 (100.0)
F	6	4 (66.7)	6 (100.0)	6 (100.0)
G	6	5 (83.3)	6 (100.0)	5 (83.3)
H	6	6 (100.0)	5 (83.3)	6 (100.0)
TOTAL	**41**	**38 (92.7)**	**36 (87.8)**	**39 (95.1)**

4.6. Confirmation of identified bacteria using *phoA* and 16S rDNA PCR assay

A total 150 *E. coli* isolate were identified from the 12 sample in 6 stations. However, only 53 presumptive *E. coli* strains were randomly picked from different estuary stations (A, B, D, E, G, and H), (water=50; sediment= 3 isolates) for the further molecular work due to limited resources. A monoplex PCR assay was performed to detect the *E. coli* housekeeping gene (*phoA)* on all 53 presumptive *E. coli* isolates to confirm the species. All the 53 *E. coli* isolates which were positive by CHROMagar^{TM} Orientation, biochemical tests and Biolog Gen III microplate System test, showed the presence of *phoA* gene (Fig 4.9). DNA sequencing analyses confirmed that the presumptive isolates were indeed *E. coli* (99% homology) Appendix 5.

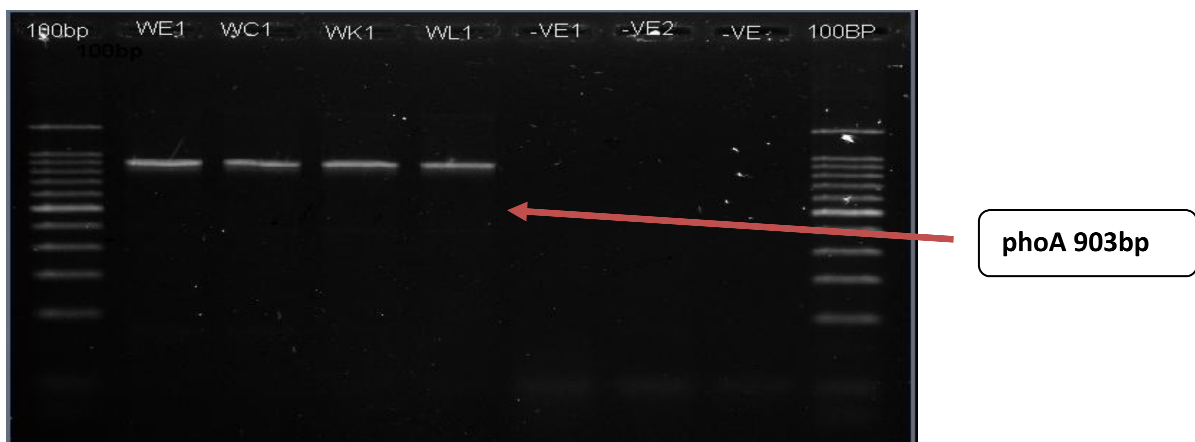


Fig 4.9. Representative gel of monoplex PCR for *phoA* gene present in all the presumptive *E. coli* isolates.

L_1: Presumptive *E. coli* isolates from station H; C_1: Presumptive *E. coli* isolates from station G; K_1: Presumptive *E. coli* isolates from station B; E_1: Presumptive *E. coli* isolates from station D. Confirmed *E. coli* isolate by previous student was used as positive control. –VE1; *Enterobacter aerogenes* , -VE2; *Enterococcus casseliflavus* and -VE3; Water are negative controls.

16S rDNA PCR assay was carried out to detect the 16S rRNA gene of the *Enterobacre*, *Klebsiella* and *Enterobacter* (Fig. 4.10). The bands generated were approximately 586 bp. The sequencing result of the 16S rDNA was queried by accessing NCBI through BLASTn. The result showed that the presumptive isolates were indeed *Enterococcus* (99% homology), *Klebsiella* (99% homology) and *Eenterobacter* (99% homology) Appendix 5.

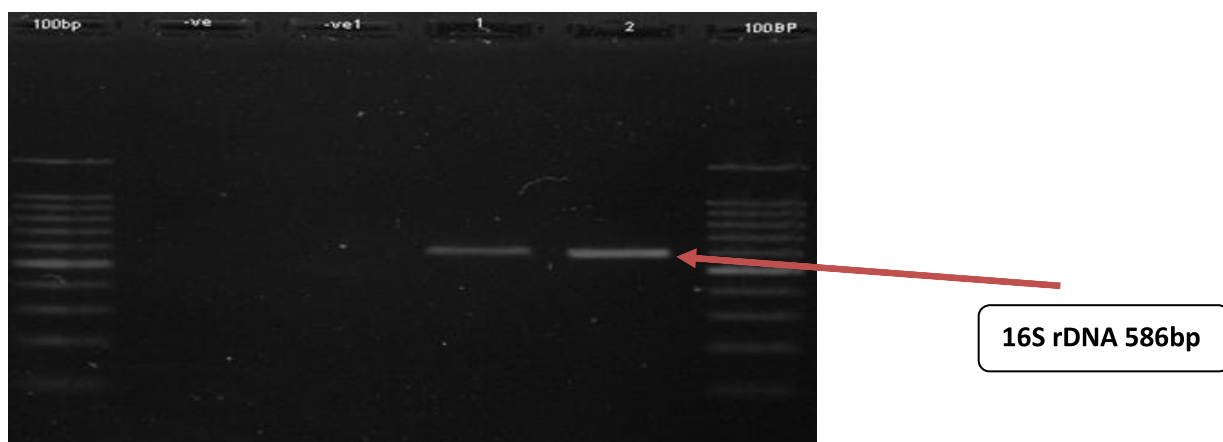


Fig 4.10. Representative gel of 16S rDNA PCR assay

1: *Enterococcus*; 2: *Klebsiella*; -VE and –VE1 are water as negative control

4.7. Genetic diversity of *E. coli* using REP-PCR

Genetic diversity of *E. coli*, as common fecal indicator bacteria along the Kuala Sepetang estuary was determined by using a molecular typing method, REP-PCR. Based on the results, a total of 38 REP-PCR banding patterns comprising of 20-30 DNA fragments were obtained by genomic diversity analysis of 53 *E. coli* isolates. The size of REP-PCR amplicons ranged from 100bp to 3000bp. All the isolates exhibited variable numbers of banding patterns However, four common bands approximately, 1550bp, 1250bp, 480bp and 400bp were observed (Fig. 4.11). For uniformity, all the bands within the window of

analysis from 250 bp to 1600 bp were scored for cluster analysis using the BioNumerics software. The REP-PCR amplification was repeated two times to evaluate the reproducibility of this technique. The results showed that the banding patterns were reproducible.

Based on dendrogram, all the 53 *E. coli* isolates were grouped into 9 clusters (I, II, III, IV, V, VI, VII, VIII and IX) using a similarity coefficient of 85%, although one isolate (L8) did not cluster with any other isolates(Fig. 4.12). Cluster III was major cluster consisting of 41% (n=22/53) of total isolates, was subdivided into two clusters [III (1) and III (2)], Cluster III (1) consisted of 15 isolates were collected from stations D and E. This indicates that most of isolates from these two stations may have originated from a similar linage. Interestingly 46% (n=7) of *E. coli* isolates in station E and 13% (n=2) in station D from this cluster shared the same fingerprinting patterns. Cluster III (2) consisted of 7 isolates that were from stations G, D, E and H. Two isolates from station E harboured the same REP-PCR pattern. Thus, this cluster showed that diversity of *E. coli* isolates in station E is very low and they mostly share the same DNA genomic patterns.

Cluster IX consisted of 8 isolates from stations B, G and H and these isolates originated from the same clonal linage of *E. coli*, this conception could be found within these three stations in clusters IV and VII which consisted of 7 isolates (isolated from B and H) and 4 isolates (collected from H, G and A), respectively (Fig. 4.12).

Some isolates from the same station source were clustered together, such as clusters VI and VIII which were consisting of two isolates each, the isolates were collected only from station A. cluster I included three isolates which were isolated from station H. Furthermore, these three clusters included two isolates each, which were not

distinguishable as they had same REP-PCR pattern. Interestingly cluster II and V included two isolates each, with 90% and 89% similarity, respectively, in spite of geographical distance. Thus, clustering of these isolates was probably associated on the geographical locations which are interconnected to each other.

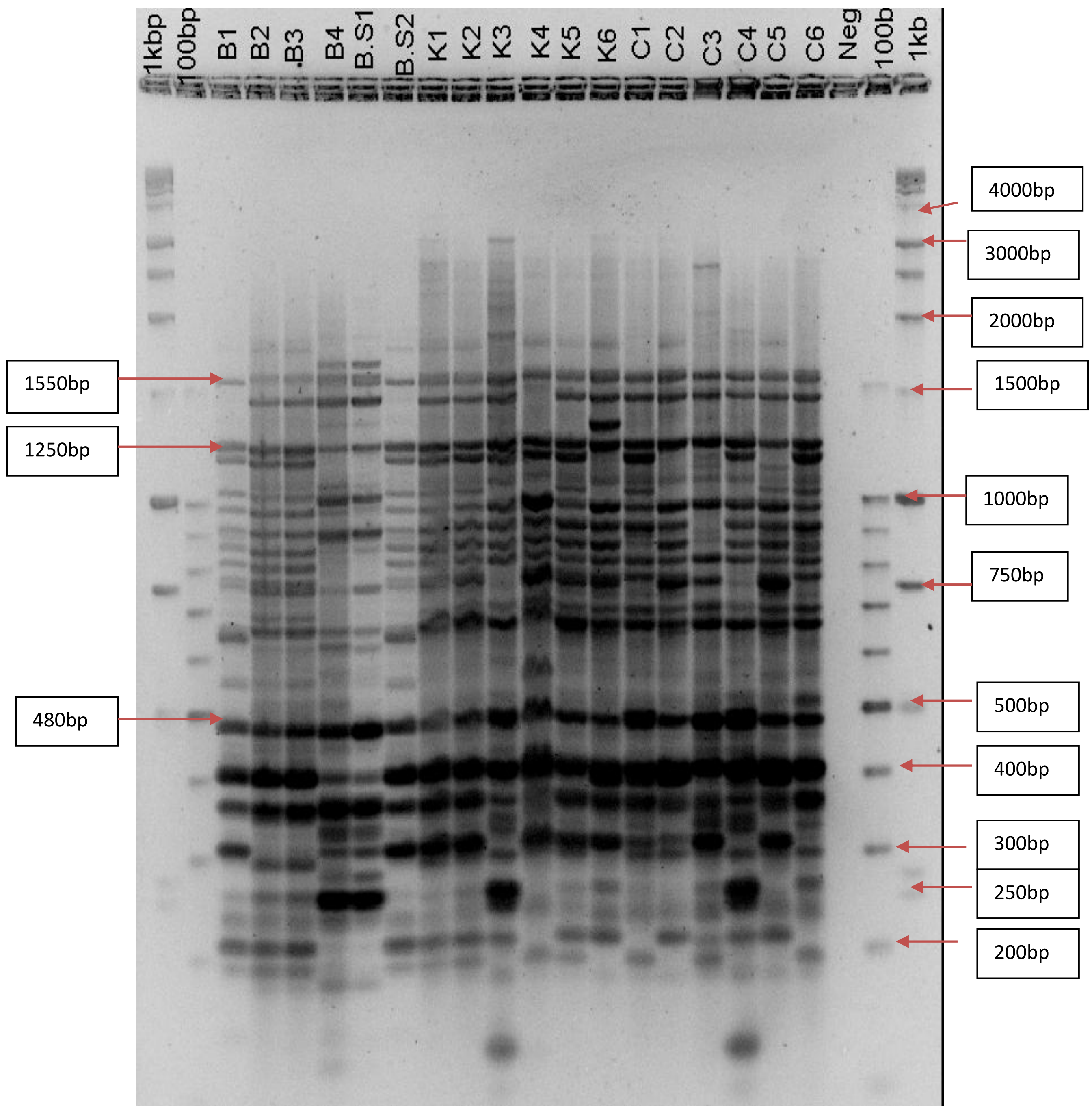


Fig. 4. 11. Representative of Rep-PCR gel using REP primer. , B1, B2, B3, B4, B.S1 and B.S2 were isolated from station A; K1, K2, K3, K4, K5 and K6 were isolated from station B; C1, C2, C3, C4, C5 and C6 isolated from station G.

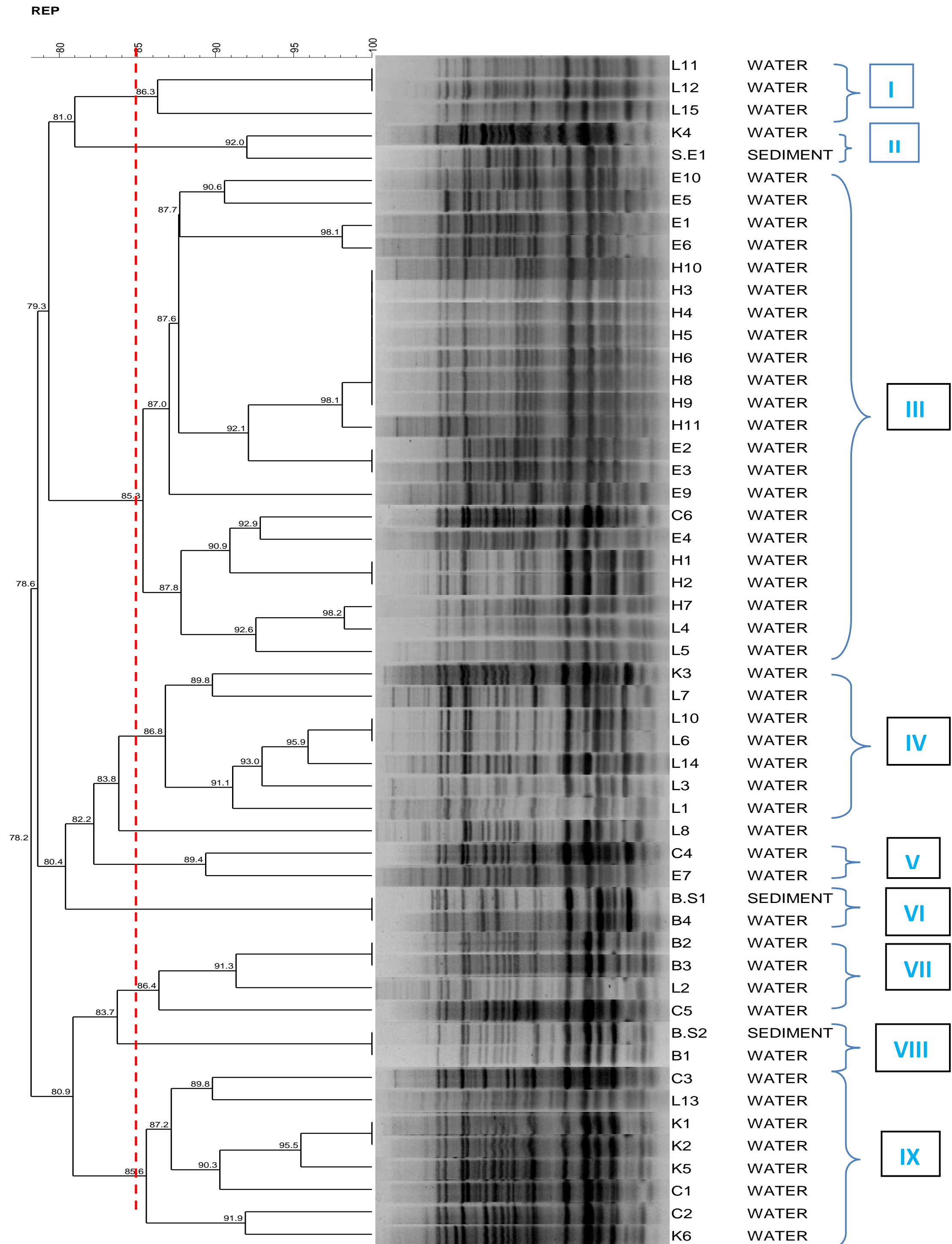


Fig 4. 12. Dendrogram showing the cluster analysis of the REP-PCR patterns from 53 isolates of estuarine *E. coli* generated by BioNumerics software. All patterns clustered into 9 clusters (I, II, III, IV, V, VI, VII, VIII and IX).

Isolates were collected from stations A, B, D, E, G and H. (B1, B2, B3, B4, B.S1 and B.S2 from station A; K1, K2, K3, K4, K5 k6 were isolated from station B; C1, C2, C3, C4, C5 and C6 from station G; E1, E2, E3, E4, E5, E6, E7, E8, E9, S.E1 and E10 from station D; H1, H2, H3, H4, H5, H6, H7, H8, H9, H10 and H11 from station E; L1, L2 L3, L4, L5, L6, L7, L8, L10, L11, L12, L13, L14 and L15 were isolated from station H).

CHAPTER 5

DISCUSSION

5.1. Culturable estuarine bacterial population on CHROMagar™ Orientation

The CHROMagar™ Orientation cultivable bacteria population, which were characterized by only four general morphotypes, changed gradually along the salinity gradient in the estuary (Fig.4.3 & Fig.4.4). The gradual change in the CHROMagar™ Orientation cultivable bacteria population along the salinity gradient was more obvious in the sediment population. The cultivable bacteria population in estuarine waters was subjected to greater fluctuation, which makes sense as the environmental parameters in the surface water changes more drastically than at the bottom. The predominant CHROMagar™ Orientation morphotypes in both the water and sediment samples along the estuary were the green and colorless morphotypes, except for in the estuarine waters from station D, E and F (Fig.4.3). Interestingly, the green morphotype was more dominance in the sediment of upper estuary (station A, B and G); whereas the colorless morphotype dominated the bottom sediment of lower estuary (station C, D, E and F) (Fig.4.4). Identification of representative isolates of green morphotype (n=107) using Biolog Gen III Microplate System and biochemical assays showed that the green morphotype comprised only of firmicutes, including *Enterococcus* sp., *Exiguobacterium* sp., *Paenibacillus* sp., *Stenotrophomonas* sp. and *Staphylococcus scirius* (Table 4.4). The colorless morphotype was mainly predominated by *Pseudomonas* sp., *Acinetobacter* sp. and *Hafnia* sp. (Table 4.4). The blue and purple morphotypes were accounted less frequently in the estuary. The blue morphotype were found to be composed mainly of *Bacillus sp.*; while *Aeromonas* sp.,

E. coli, Citrobacter sp., *Serratia* sp., *Vibrio* sp. and *Staphylococcus hominis* made up the purple morphotype. However, the occurrences of *E. coli* and *Citrobacter spp.* were extremely low.

5.2. Low recovery of *Enterobacteriaceae* by direct plating on CHROMagar^{TM} Orientation

Direct plating of estuarine water and sediment samples on CHROMagar^{TM} Orientation yielded a high variety of bacteria species of various colors and colony morphologies. However, in this study, we found that direct plating of estuarine samples on CHROMagar^{TM} Orientation produced low recovery of *Enterobacteriaceae,* specifically *E. coli, Klebsiella* and *Enterobacter*. Direct plating of the estuarine samples on CHROMagar^{TM} Orientation gave yield to predominantly Gram-positive bacilli, Gram-positive cocci (*Enterococci*), and marine-associated species (*Aeromonas, Vibrio* and *Stenotrophomonas*). The animal and human hosts are the natural habitat of *Enterobacteriaceae*. Exposure of these bacteria into the estuaries with unfavorable conditions is believed to induce injury and stress to the cells and therefore cause a low recovery on agar medium (Mouslim *et al*., 2002; Rozen and Belkin, 2001; Winfield and Groisman, 2003). This could also explain why direct plating of estuarine samples on CHROMagar^{TM} Orientation in this study produced mainly Gram-positive bacteria and only some Gram-negative bacteria that are halophilic or halotolerant.

To prove the speculation, an extra step of pre-enrichment in buffered peptone water for 4 hours and 12 hours was employed before plating onto CHROMagar^{TM} Orientation. The enrichment was employed based on target bacteria which were *Enterobacteriaceae* family, so buffered peptone water was used as enrich medium. As expected, this pre-

enrichment step in rich nutrient media had resulted in enumeration of *E. coli, Klebsiella, Enterobacter* and *Citrobacter*, indicating pre-enrichment is necessary to resuscitate the injured cells (Fig 4.2).

CHROMagar™ Orientation is claimed to have a broader application as a general nutrient agar for the isolation of various microorganisms, besides its application in detection of only urinary tract pathogens. However, the chromogenic mix in the media that enable the differentiation of bacteria based on color formation might have a certain degree of inhibitory effect on some bacteria and injured cells. Therefore, direct contact of bacteria cells, specifically those injured or stressed cells to the media will impose more stress to the cell. Consequently, injured or stressed cells are inhibited or inactivated and cause a low recovery. Thus, to prove this speculation, the water and sediment samples were filtered through a 0.45µm membrane before placed it on CHROMagar™ Orientation. The membrane filter acts as a protective layer to avoid direct contact of bacteria cells to any inhibitory agents might be presence in the agar. As a result, membrane filtration approach was proved to be a better method than direct plating for bacteria enumeration as it supported growth of *Enterobacteriaceae* and also the other Gram-positive bacteria and marine associated bacteria. Also, direct plating of samples on CHROMagar™ Orientation yielded lesser count as compared to direct plating on general nutrient agar (Table 4.3).

5.3. Isolation and identification of fecal indicator bacteria and potential human pathogens from estuarine waters and sediments using CHROMagar™ Orientation

.More than half (61.2%) of the bacteria that were isolated in this study, was one of the three EPA recommended fecal indicators of health risk in salt waters and fresh waters (total coliforms, *E. coli* and *Enterococci*) (EPA, 2003).

E. coli (n=150) was the predominant bacteria species isolated from the estuarine waters and sediments using CHROMagar^{TM} Orientation. All of the isolates identified to be *E. coli* produced a red violet colored colony with red violet halo around the colony on CHROMagar^{TM} Orientation, which is easily distinguishable from other bacterial species (Appendix 5). Hundred fifty presumptive *E. coli* strains CHROMagar Orientation were confirmed by using Biolog Gen III. Fifty three *E. coli* strains were randomly picked and subjected to the PCR. All fifty three presumptive *E. coli* reconfirmed as *E. coli* strains. The color and morphology of estuarine *E. coli* on CHROMagar^{TM} Orientation observed in this study was no different to those of clinical-origin as reported previously (Merlino *et al.*, 1996; Samra *et al.*, 1998). The blue/violet blue colonies isolated from CHROMagar^{TM} Orientation in this study were identified to be one of three bacterial genus, namely *Enterobacter* spp. (n=20), *Klebsiella* spp. (n=110) and *Raoultella* spp. (n=12). *Enterobacter aerogenes* and *Enterobacter clocae ss dissolvens* were the only two species of *Enterobacter* genus isolated; while *Klebsiella oxytoca* and *Raoultella planticola/ orthinolytica* were the only species of *Klebsiella* and *Raoultella* genus isolated, respectively. *Enterobacter* sp. produced pale blue colored colony with or without clear edge, which was distinct from colony of *Klebsiella* sp. and *Raoultella* sp. That was violet-blue to metallic in color. Both, *Klebsiella* sp. and *Raoultella* sp. were non-distinguishable on CHROMagar^{TM} Orientation. In fact, the genus *Raoultella* is composed of Gram-negative, oxidase-negative, aerobic, nonmotile, capsulated, facultatively anaerobic rods, which was formerly classified as *Klebsiella* (Drancourt *et al.*, 2001). It consists of only three species: *Raoultella ornitholyca*, *Raoultella planticola* and *Raoultella terrigena*. Of 520 bacteria isolated in this study, only 2 isolates were identified as *Citrobacter*. We were able to identified only one of the isolates to its species level (*Citrobacter koseri/ youngae*)

using Biolog gen III microplate System. *Citrobacter* produced red-violet to reddish purple colony with pale yellow edge on CHROMagar^{TM} Orientation. Even though *Citrobacter* (Specifically *Citrobacter freundii*) were reported to regularly demonstrate a metallic blue to violet colony in both studies carried out by Merlino and co-workers (1996) and Samra and co-workers (1998), none of the metallic blue colored colony was identified to be *Citrobacter* in my study. In this study, four isolates demonstrating blue violet colony with red violet colored edge that deepened with prolong incubation at room temperature were identified to be *Serratia marcescens* marcescen. The identification of *Serratia marcescens* was reconfirmed by sub-culturing on general nutrient agar, in which it produces orange to red pigmented colony. *Serratia liquefacicnes* and *Serratia marcescens* were reported in the work of Merlino and co-workers (1996) to produce light aqua blue colony that may darken to navy blue with pigment production at room temperature. The red violet colored edge of *Serratia* colony that was observed in this study was not reported in Merlino and co-workers' study (Merlino *et al*., 1996). In this study, only one isolate of *Hafnia alvei* was obtained. On CHROMagar^{TM} Orientation, the colony was white with sheer red-violet pigmentation in the center of the colony. No report could be found on the color and morphology of *Hafnia* sp. on CHROMagar^{TM} Orientation.

Interestingly, CHROMagar^{TM} Orientation serves as a good media for enumeration and isolation of *Enterococci* (without enrichment) in estuarine water and sediment. On CHROMagar^{TM} Orientation, *Enterococci* grew as pin point isolated green blue colony (0.1-0.2 mm in diameter) with green blue diffused halo around the colony. The distinctive colony morphology of *Enterococci* on CHROMagar^{TM} Orientation was found to be consistent, regardless of sample types (Merlino *et al*., 1996; Samra *et al*., 1998). In this study, *Enterococci* were abundant in the surface water and sediment of Kuala Sepetang

estuary. Of the 22 isolates demonstrating pinpoint green blue colony, all were identified as *Enterococcus caselliflavus*.

The remaining 199 isolates (38.0%) which are not commonly used as fecal indicators belong to 5 genera of Gram negative bacteria (*Acinetobacter, Aeromonas, Pseudomonas, Stenotrophomonas* and *Vibrio*) and 4 genera of Gram positive bacteria (*Bacillus, Exiguobacterium, Paenibacillus* and *Staphylococcus*) (Table 4.4). The most prevalent within this group was *Aeromonas* (n=46). As many as five species of *Aeromonas* were isolated in this study. The colony morphology of *Aeromonas* on CHROMagar™ Orientation was very much similar to *Citrobacter* and most of the time non-distinguishable. However, *Aeromonas* sp. regularly produced larger colony (1.5-2.5 mm in diameter) on CHROMagar™ Orientation as compared to *Citrobacter* sp. (0.5-1.5mm). Actually, *Citrobacter* sp. and *Aeromonas sp.* produced red violet colony on CHROMagar™ Orientation, which was very much closed to those produced by *E. coli*. The key characteristic in differentiating *E. coli* from *Citrobacter* and *Aeromonas* was the presence of red violet diffusion formed in the agar around the *E. coli* colony. Both *Citrobacter* sp. and *Aeromonas* sp. were not able to produce red violet diffusion around the colony, even with prolong incubation at room temperature or at 37°C up to 3 days.

Twenty-four isolates with irregular light green colony on CHROMagar™ Orientation were picked for further identification. All of these isolates were identified as *Stenotrophomonas rhizophila*. Colorless colonies on CHROMagar™ Orientation were isolated and identified as either *Acinetobacter johansonii* or *Pseudomonas putida*. *Acinetobacter johansonii's* colony was milky white in color and slightly smaller in size (1.0-1.5mm) as compared to colony of *Pseudomonas putida* (2.0mm) which was transparent on. Twenty-seven isolates of *Vibrio cholorae* and *Vibrio fluvialis* were isolated.

These isolates produced unique peacock blue/ red violet colony: red violet colony turn into blue-green edge over prolong incubation. This green colored edge will only developed over prolong incubation for 48 hours at 37°C.

Other than Gram-negative rod-shaped bacteria and *Enterococci,* direct inoculation of estuarine water and sediment samples on CHROMagar^{TM} Orientation yielded also *Staphylococcus* sp., some Gram-positive *bacilli,* including *Bacillus* sp. (n=71), *Paenibacillus* sp. (n=5) and *Exiguobacterium* sp. (n=8). *Staphylococcus sciuris, Staphylococcus hominis* and *Staphylococcus saprophyticus* were isolated from the estuarine waters. These three species of *Staphylococcus* grew as small pulvinate (0.5mm in diameter), green blue (*Staphylococcus scirius*) and red violet colored colony on CHROMagar^{TM} Orientation. Unlike colony of *Enterococci,* colored diffusion around the colony was not observed in *Staphylococcus*. Direct enumeration of estuarine waters and sediments on CHROMagar^{TM} Orientation yielded a significant amount of rough waxy colonies of various colors, including light blue, green, yellow, white and colorless. Seventy-one rough waxy colonies isolated were identified as *Bacillus megaterium, B. slarius, B. pumilis, B. cereus/ pseudomycoides* and *B. cereus/ thurigiensis.*

Surprisingly, eight isolates of *Exiguobacterium aurantiacum* and *Exiguobacterium acetylicum* were isolated only from estuarine waters at station F located along river Kuala Sangga Besar. *Exiguobacterium* sp. produced light green/ glue-green colony with broad colorless or yellow colored edge on CHROMagar^{TM} Orientation. They yielded yellow pigmented colony when grew on general nutrient agar. *Exiguobacterium* sp. is alkaliphilic, halotolerant, non-spore-forming, low G + C, Gram-positive facultative anaerobes (Pitt *et al*., 2007; Vishnivetskaya *et al*., 2009). *Exiguobacterium* sp. have been isolated from markedly diverse sources, including ancient Siberian permafrost, Greenland glacial ice, hot

springs at Yellowstone National Park, the rhizosphere of plants, the environment of food processing plants and also from patients (Funke *et al*., 1997; Vishnivetskaya *et al*., 2009; Pitt *et al*., 2007; Zijnge *et al*., 2003). Both species of *Exiguobacterium* isolated from this study have been identified as the aetiological agent of bacteremia in a neonate (Pitt *et al*., 2007), adults (Pitt *et al*., 2007; Zijnge *et al*., 2003) and elderly (Keynan *et al*., 2007). Most of the media used for microbiological monitoring of rivers and coastal regions, for instance EMB, MEndo, mFC agar for detection of coliforms and *E. coli* do not support the growth of *Exiguobacterium. Exiguobacterium* was generally isolated on general media (e.g. marine agar and nutrient agar). However, isolation of *Exiguobacterium* from samples with high microflora background is very difficult as the presence of a high level of background microflora and competitor organisms could mask the presence of *Exiguobacterium* which is likely the minority species in the population. The use of CHROMagar^{TM} Orientation which allows on-the-plate differentiation and identification of various bacteria presence in the sample increases the chance to detect and isolate this interesting bacterium. Enumeration of the coliform bacteria base on using CHROMagar^{TM} Orientation was not conducted in the current study due to analysing the efficiency of this medium whether it suitable to be used for environmental samples. Therefore, it may be used in the future work.

5.4. Comparison of the performance of CHROMagar^{TM} Orientation, CHROMagar^{TM} ECC, EMB and MacConkey agar for accurate detection of fecal indicators in estuarine samples

As result showed that CHROMagar^{TM} Orientation, in comparison with EMB and CHROMagar^{TM} ECC, yielded a good presumptive detection of fecal bacteria, *Enterococcus* and coliforms (*E. coli, Klebsiella, Enterobacter* and *Citrobacter*) simultaneously (Table.

5.1.), in which all of these bacteria are recommended by EPA as fecal indicator of health risk in salt waters and fresh (EPA, 2003). At the same time, some other non-fecal pathogen such as *Vibrio* sp, *Acenitobacter* sp., and *Pseudomonas* sp., could easily isolated and identified on the CHROMagar^{TM} Orientation. The purpose of using CHROMagar^{TM} ECC is in differentiation of *E. coli* from other coliforms base on using chromogenic substrate in CHROMagar^{TM} ECC by target microorganisms. However, the differentiation between coliform cannot be achieved. In this study, some isolates of *E. coli* could not be detected by CHROMagar^{TM} ECC; while bacteria other than coliform and *E. coli* showed the same colony morphology on CHROMagar^{TM} ECC. EMB was another medium which was compared to CHROMagar^{TM} Orientation in simultaneous presumptive detection of fecal bacteria and non-fecal bacteria (Table 5.1). EMB is mostly used to isolate the Gram-negative bacteria (Levin *et al*., 1918). On the other hand, the differentiation between most of Gram-negative bacteria was difficult due to resulting in the same color and colony characteristic. Growth of *Enterococcus* as important fecal indicator bacteria in water (EPA, 2003) was limited in both EMB and CHROMagar^{TM} ECC.

The accuracy of CHROMagar^{TM} Orientation was evaluated in contrast to EMB and CHROMagar^{TM} ECC in presumptive detection of *E. coli* (Table.4.5). EMB mechanism in detection of pathogenic and no pathogenic microorganisms is depended on production of acid resulting from lactose fermentation. *E. coli* produce strongly acidic condition during growth on EMB, this acidic condition gives a distinctive metallic green (Horvath *et al*., 1974). However, some species of *Enterobacter* sp., appear with the same colony morphology on EMB (Horvath *et al*., 1974). This study showed that the accuracy of EMB was 88.1% in detection of *E. coli* (Table 4.6) and the accuracy of CHROMagar^{TM} ECC and CHROMagar^{TM} Orientation was 95.2% and 92.9%, respectively. Although the

CHROMagar^{TM} ECC showed the highest accuracy, some *E. coli* strains could not be detected on CHROMagar^{TM} ECC based on the CHROMagar^{TM} ECC mechanism in which all presumptive *E. coli* isolates should appear as green/ blue colony on CHROMagar^{TM} ECC due to the presence of β-D glucuronidase and β-glucuronidase enzymes. β-glucuronidase is present in coliform bacteria and β-D glucuronidase is present in all *E. coli* strains expect *E. coli O157:H7* (Conda, 1960). All *E. coli* strains appear in blue-green color but *E. coli O157:H7* colonies appear in colorless to pink colonies. Due to CHROMagar^{TM} Orientation mechanism bacterial can be detected on CHROMagar^{TM} Orientation based on producing enzyme for metabolism of lactose and glucosides or both. *E. coli* could differentiate by its red violet color colony with red violet diffusion resulting lactose metabolism in CHROMagar^{TM} Orientation (Becton, 2008).

The accuracy of CHROMagar^{TM} Orientation was compared with MacConkey and EMB media in detection of coliform bacteria (*Klebiella* sp and *Entrobacter* sp) (Table 4.7). The identification of coliforms on both MacConkey and EMB media is based on lactose fermentation (Horvath *et al*., 1974; Merlino *et al*., 1996). Based on that, the accuracy of MacConkey and EMB in presumptive identification of coliform was 87.8% and 92.7%, respectively (Table 4.6). CHROMagar^{TM} Orientation with highest accuracy 95.1% showed that it can be used as desirable media in presumptive detection of coliforms (*Klebiella* sp and *Entrobacter* sp).

Table 5.1. Comparison of CHROMagar^{TM} Orientation , CHROMagar^{TM} ECC and EMB in simultaneous presumptive identification of bacteria

	Bacteria genus	CHROMagar^{TM} Orientation	EMB	CHROMagar ^{TM} ECC
Coliform	*Citrobacter*	Red-violet with white to pale yellow	"fish eye" like colony with black center (some are black colony with green sheen)	Mauve to Red
	Enterobacter	Blue	"fish eye" like colony with black center (some are black colony with green sheen)	Mauve to Red
	Klebsiella	Blue violet with colorless edge	fish eye like colony with black center	Mauve to Red
	Serratia	Violet with Red violet edge	fish eye like colony with purple center	Red
	Hafnia	White (with sheer Red-violet pigmentation)	fish eye like colony with black center	Light pink
Fecal coliform	*E. coli*	Violet-red with Pale violet-red edge and diffusion	Black colony with green sheen (some are colorless)	Blue (some are white)
Other FIB	*Enterococcus*	Green-blue with Green-blue diffusion	Partially inhibited (pinpoint colorless colony)	Inhibited
Non-coliform waterborne pathogen	*Aeromonas*	Red-violet with Colorless/ Pale yellow	Partially inhibited (pinpoint colorless colony	Inhibited
	Acinetobacter	Colorless (milky in color)	Purple with colorless edge	Clear transparent
	Pseudomonas	Colorless	colorless	Colorless
	Vibrio	Transparent with pink center (turn into blue-green over prolong incubation)	Colorless	Inhibited

5.5. Genetic diversity of *E. coli* using REP-PCR

Molecular subtyping tools are used to study the clonal and close relationship between isolates within a species. REP-PCR produces DNA fingerprints which allow discrimination of bacteria strains (Versalovic *et al*., 1991; Woods *et al*., 1993).The possible spread of bacteria is one of the important issues because they can be dispersed in the environment through tidal activity (Mekalanos, 1999). Thus, it is possible to identify source of pathogen or to pursue the regional and global spread of pathogens using this method to take right decision for monitoring of water pollution.

REP-PCR as the DNA fingerprinting technique was used in this present study to determine the genetic diversity of *E. coli* species in Kuala sepetang estuary. *E. coli* was selected to further study for their genetic diversity in this work was due to several factors. Firstly, *E. coli* is one of the most commonly used fecal indicator in the water research; secondly, *E. coli* was widely distributed along the Kuala Sepetang estuary; thirdly, the identity of *E. coli* could be easily re-confirmed using the developed molecular approach in this study. Therefore, E. coli was used as the model species to study the possible fecal contamination in Kuala Sepetang estuary. *E. coli* isolates were highly diverse in this environment. The *E. coli* isolates were collected in different locations (stations A, B, C, D, E, F and G) in Kuala Setetang estuary to provide a better understanding of the population structure obtained

The resultant dendrogram showed that diversity of *E. coli* was high in the upper estuary (A, B and G) which was close to the village. Furthermore, this diversity was very high in Kuala Selinsing River in which stations G and H are located. However genetic diversity of *E. coli* increased along the river with a concomitant decrease in temperature, pH and salinity (Table 4.2). *E. coli* isolates which were collected from stations G and H

were separated in four (III, V, VII and IX) and five clusters (I, III, IV, VII and IX), respectively (Fig 4.12).

On the other hand, this diversity decreased along Kuala Sangga Besar with increasing of temperature, pH and salinity in which stations B, D and E were located. All the *E. coli* strains those were isolated from station E which was close to the open ocean water with high salinity, temperature and pH, were grouped in cluster III. As diversity of *E. coli* decreasing along Kuala Sangga Besar River, it was expected that this decline should have appeared in station D as it was following of station B (*E. coli* isolates in both stations B and D were separated into three clusters), but since station D was closed to the other village (Fig 4.1) and another river joined to this sampling site, it effected on *E. coli* diversity in this sampling site.

In this study *E. coli* isolates were highly diverse in Kuala Selinsing River, especially in station H. In a study which was done by Anderson *et al* (2006) in Florida, USA. He reported that the diverse and unstable nature of *E. coli* populations was found is due to the survival rate of *E. coli* strains in environmental waters are significantly different according to the strain characteristics where the survival rate is depended on the environmental condition and some of them exhibit prolonged persistence in environmental waters (Anderson *et al*; Kon *et al*., 2007). Furthermore, it can be explained that some of the *E. coli* strains which are adapted to the environment are also recognized to be widespread in environment, especially in tropical environment (Kon, 2009).One possibility that can affect on *E. coli* diversity in Kuala Selinsing River, especially in station H was presence of plenty of seagulls during sampling time (personal observation, Appendix 3). These birds can act as a vector to deliver *E. coli* to the environment either directly or via tributaries (Kon, 2009).

Since the water is streaming from upstream to downstream of estuary and due to geographical location of the water which is in interconnected in Kuala Spetang estuary most of the isolates clustered together, event from different Rivers (Kuala Selinsing and Kuala Sangga Besar Rivers). As Kon *et al*. (2007) reported that different strains clusters from the same sampling location were closely related due to deposition of *E. coli* from a single source and the other possibility for presence of related strains is due to survival and growth of *E. coli* strains in environment. It has been reported that some *E. coli* strains are more resistance in sediment and water in subtropical and tropical environment (Kon *et al*., 2007).

CHAPTER 6

CONCLUSION

From this work, it was demonstrated that CHROMagar™ Orientation allows simultaneous isolation and on-the-plate differentiation of *Enterobacteriaceae* and other various waterborne pathogens and immediate characterization of general population composition in the estuarine sample. The cultivable bacterial population on CHROMagar™ Orientation might not be useful in the microbial ecology study. Nevertheless, it provides a rapid (within 24 hours), economical and easy approach to detect changes in the bacterial population, which could be due to a drastic change in the environment.

Moreover, CHROMagar™ Orientation appears to be useful in rapid screening for bacterial fecal indicators, namely coliforms and *E. coli* with 95.1 % and 92.9% accuracy, respectively. It shows that could be used to easily identify *Enterococcus* spp as it is suggested to be better indicator of fecal pollution in coastal and estuarine environments, because their better survival in saline waters. Therefore, using this medium could diminish the multiple required plates to identify *E. coli*, coliform (such as *Klebsiella*, *Seratia*, *Enterobacter*) and *Enterococcus* sp.

This study shows that REP-PCR is a useful and simple molecular subtyping tool to determine the gene diversity of *E. coli* along Kuala Sepetang estuary. However the small number of isolated *E. coli* was a drawback and there was fluctuation in the physicochemical properties in estuary, thus, these data could not present the exact genomic diversity of *E. coli* in the Kuala Sepetang estuary. Furthermore the result should be evaluated to find out the exactly source of contamination in this environment.

Kuala Sepetang estuary, as one of the major estuaries supporting numerous aquaculture activities, was found to be contaminated with various types of waterborne bacterial pathogens and therefore pose a high risk to the public.

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