Faculty of Resource Science and Technology

Genetic Diversity of *Escherichia coli* isolated from Birds and Bats by using Pulsed Field Gel Electrophoresis (PFGE)

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Bachelor of Science with Honours
(Resource Biotechnology)
2012

ACKNOWLEDGEMENT

First and foremost, thank to God for his blessing in completing this project. I am deeply grateful and would like to express my appreciation to my supervisor, Prof. Kasing Apun for her advice, support, guidance, and countless hours she had spent for me and my project. Besides that, I would like to thank master students, Miss Kho Kai Ling for her valuable time helping me in my laboratory works. To my friends, especially my coursemates in Resource Biotechnology, thank you for the friendship and for supporting each other in completing our project. Not forgetting also my lovely parents and family members that always encourage and understand me. Thanks for always by my side to support me all the way. Last but not least, I would like to thank Faculty of Resource Science and Technology (FRST), Unversiti Malaysia Sarawak for providing facilities and education for me.

Table of Contents

List of Abbreviations

BSA	Bovine serum albumin
CgA	Clonal group A
CSB	Cell Suspension Buffer
CLB	Cell lysis buffer
CLRW	Clinical laboratory reagent water
DNA	Deoxyribonucleotide acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	*Enterohemorrhagic E. Coli*
OD	Optical density
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Randomly amplified polymorphic DNA
RE	Restriction enzyme
SARS	Severe Acute Respiratory Syndrome
STEC	Shiga toxin *E. coli*
TE	Tris- EDTA
UT	Untypeable
UTI	Urinary tract infection
VTEC	Verocytotoxin producing *E. coli*

Lists of Tables

Lists of Figures

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ABSTRACT

Escherichia coli (*E. coli*) belongs to the family of Enterobacteriaceae. *E. coli* can be found in animals such as birds and bats which cause zoonoses. In this study, a total of 22 isolates of *E. coli* were used which 11 isolates from birds in Kuching; 6 isolates from broiler chicken and 5 isolates from soil while another 11 isolates were bats from different habitats; 2 isolates from Bukit Aup Sibu, 3 isolates from Nanga Merit Kapit, 4 isolates from Kubah National Park and 2 isolates from Matang Wildlife Centre. The *E. coli* isolates digested with *Xba*I generated 6 to 13 bands with sizes of approximately 54.7-668.9 kb. The PFGE profiles were further analysed by using the RAPDistance program and a dendrogram was constructed based on the difference of genetic distance. In this study, the genetic diversity of *E. coli* isolated from birds and bats was determined from the analysis. Among these samples, the isolates from broiler chicken and soil were more diverse compared to isolates of bats.

Keywords: *Escherichia coli*, birds, bats, *Xba*I, Pulsed Field Gel Electrophoresis

ABSTRAK

Escherichia coli (E. coli) *merupakan salah satu Enterobacteriaceae*. *E. coli* sering dikaitkan dengan burung dan kelawar yang boleh menyebarkan penyakit berjangkit kepada manusia. *Dalam kajian ini, sebanyak 22 isolat* E. coli *yang digunakan. 11 isolat adalah daripada spesis burung dari kawasan Kuching; 6 isolat daripada ayam dan 5 isolat daripada tanah. Manakala 11 lagi isolat daripada kelawar dari pelbagai habitat; 2 isolat dari Bukit Aup Sibu, 3 isolat dari Nanga Merit Kapit, 4 isolat dari Taman Negara Kubah dan 2 isolat dari Taman Haiwan Matang. Isolat* E. coli *yang dipotong dengan menggunakan enzim* XbaI *telah menghasilkan 6-13 jalur* DNA *dengan saiz 54.7-668.9 kb. Profil* DNA *ini telah dianalisis dengan menggunakan program* RAPDistance *dan* dendrogram *telah dibentuk berdasarkan jarak genetik. Dalam kajian ini, kepelbagaian genetik* E. coli *daripada spesis burung dan kelawar dapat dipastikan dengan analisis tersebut. Isolat daripada ayam dan tanah menunjukkan lebih kepelbagaian jika dibandingkan dengan isolat daripada kelawar.*

Kata kunci: Escherichia coli, *burung, kelawar,* XbaI, Pulsed Field Gel Electrophoresis

INTRODUCTION

1.1 Introduction

Almost all emerging infectious diseases of humans are zoonotics and most of it originates from wildlife (Rhyan *et al*., 2010). Wildlife reservoirs have long been recognized as the major contributor for several zoonotic diseases such as plague, rabies and tularemia (Rhyan *et al.,* 2010; Bonnedahl, 2011). According to World Health Organization (2011), millions of people were infected by zoonotic diseases in every year.

Birds have been known to act as a vector for pathogenic microorganisms that could bring diseases to humans. The example of diseases that are associated with birds are influenza virus, West Nile fever and Lyme's disease (Albureesh *et al.,* 2007; Bonnedahl, 2011). Influenza A viruses are responsible for highly contagious acute illness in humans, pigs, horses, marine mammals and birds (Bengis *et al*., 2004). Birds are known to have the enteric pathogens such as *Campylobacter* spp., *Salmonella* spp., and toxin-producing strains of *Escherichia coli* (Bonnedahl, 2011). According to Wallace *et al* (1997), the bacteria isolated from the birds were due to their feeding habits such as from rubbish, sewage outfalls, consumption of contaminated water and sewage sludge to land.

E. coli is normal flora of intestinal tract but some strain such as *E. coli* 0157:H7 is one type of pathogenic strain. The most popular zoonotic members of *E. coli* is the verotoxin-producing *E. coli* 0157:H7 (Wallace *et al.,* 1997; Nielsen *et al.,* 2004; Bonnedahl, 2011). There is a study about *E. coli* clonal group A (CgA) isolates that are

closely related to the clonal O15:K52:H1 group has been found in broiler chicken which may cause urinary tract infection (UTI) to humans (Jakobsen *et al.,* 2010). Farming animals such as chicken's farm could allow new pathogens especially *E. coli* to increase their numbers exponentially. This farming allows pathogens for example *E. coli* O157:H7 strains that may occur in small numbers to spread rapidly (Naicker, 2011). The intestines and environment of chicken act as reservoirs for extraintestinal pathogenic *Escherichia coli* (ExPEC) and these strains could pose a zoonotic risk because they could be transferred directly from birds to humans (Ewers *et al.,* 2009). According to Nielson *et al* (2004), there is a possibility that wild animals and birds act as vehicles for verocytotoxin producing *E. coli* (VTEC) in the transmission between the farms or supporting the persistence of VTEC infections in domestic animals.

Besides birds, bats are also one of the most widely distributed mammals in the world, and they are reservoirs or carriers of several zoonoses (Adesiyun *et al*., 2009). One example of disease that could be transmitted by bats is rabies. Bats, which represent approximately 24% of all known mammalian species, are frequently the maintenance hosts for many strains of rabies viruses (Bengis *et al*., 2004). Enteric pathogens from bats have been reported in Trinidad by Adesiyun *et al* (2009) and representing 12 species, reported 49 (13.0%) were positive for *E. coli*. According to Calisher (2006), bats have varied feeding habits such as insects, fish, blood, mammals and pollens. The feeding habits affect the types and distribution of bacteria in the bats gastrointestinal tract (Anand *et al*., 2004). The characteristics of the bats such as their particular diets, ability to fly, cyclic migration and daily movement patterns, their susceptibility to viruses make them suitable hosts of viruses, bacteria, parasites, fungi, and other disease agents (Calisher, 2006).

The use of pulsed-field gel electrophoresis (PFGE) in the molecular area has been subject to much research (Basim *et al*., 2001). According to Basim *et al*. (2001), PFGE is a powerful tool for characterizing various strains of bacterial DNA thus obtaining relevant information on genome size. PFGE also able to construct the physical and genetic map of chromosome of bacteria that are poorly understand at the genetic level as well as separating chromosomes in microorganisms, and in the long range mapping mammalian genes (Basim *et al.,* 2001). The PFGE protocol is a very suitable method for the epidemiological tool for surveillance studies of bacteria outbreaks (Durmaz *et al.,* 2009; Rounds *et al.,* 2010; Zou *et al.,* 2010). For example, PFGE has been used in studying of *Bordetella pertussis* (Advani *et al.,* 2004), *Salmonella* serotypes (Zou *et al.,* 2010) and *Escherichia coli* (Apun *et al.,* 2006).

Comparing to the classical electrophoresis, PFGE involves periodical changing of field direction so the various length of DNA react to the change at differing rates and the larger DNA fragments will be slower to realign their charge when field direction is changed. Classical electrophoresis uses single electric field which causes the biomolecules to migrate according to the mass which cause the larger DNA fragment block in the upper part as it cannot pass through.

During the last four to five decades, a tremendous amount of research has established *E. coli* among the important etiological agents of enteritis and several extraintestinal diseases such as urogenital infections, wound infections, mastitis, septicaemia and meningitis (Wasteson, 2001). Wildlife is well known in transmitted zoonotic diseases (Rhyan *et al.,* 2010). Therefore, this study was conducted in order to study the distribution of *E. coli* in bats, chicken and soil. It is important to study *E. coli* in

wildlife as it will affect human's health and also other animals. Besides that, zoonoses diseases will also affect the agriculture in the country.

1.2 Objectives

This study is undertaken with the following objectives to:

i) Determine the genetic diversity of *E. coli* isolated from birds and bats by using Pulsed Field Gel Electrophoresis (PFGE)

ii) Assess the genetic relatedness of *E. coli* isolated from bats in different habitats

LITERATURE REVIEW

2.1 *Escherichia coli (E. coli)*

Theodor Escherich, a German bacteriologist reported the isolation of a bacterium called *Bacteria coli* from a fecal sample in 1885 (Bhunia, 2008). The name was *bacterium coli* but it changed to *Escherichia coli* in honour of his name Escherich.

Escherichia coli (E. coli) belongs to *Escherichia* genus of Enterobacteriaceae family and commonly known as enteric bacteria. It is a type of gram negative rod shape bacteria. It is facultative anaerobe which means can grow with (aerobically) or without oxygen (anaerobically). The enteric bacteria survive in the gastrointestinal tract including oral cavity, oesophagus, stomach, intestines, rectum and anus of warm blooded animals and human (Wasteson, 2001; Bhunia, 2008). Some bacteria are pathogens as they can cause infections in people and some did not harm as they are commensal. Pathogenic *E. coli* strain can cause diseases such as diarrhea, hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) (Wasteson, 2001; Garcia *et al.*, 2010). HUS can cause kidney failure and death, particularly among children.

E. coli are shed into the environment through faeces, contaminated food like fruits, vegetables, contaminated water and soil. According to Wallace *et al.* (1997), meats are the common source of *E. coli* contamination especially in beef. There are 5 distinct groups of *E. coli* that can cause diarrheal disease: Enterohemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), Enteroadherent *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Enterotoxigenic *E. coli* (ETEC) (Doyle, 1987; Wasteson 2001; Bhunia, 2008).

2.1.1 Shiga toxin producing *E. coli*

Shiga toxin producing *E. coli* or always refer as EHEC group is the type of STEC causing disease in humans. Since Shiga toxin kills Vero toxin, this toxin also referred as verotoxin and it is called verotoxin-producing *E. coli* (VTEC) (Bhunia, 2008). *E. coli* Shiga toxins consist of two families which are stx1 and stx2 (Wasteson, 2001). According to Wasteson (2001), the stx1 is a homogenous group of toxins which related to Shiga toxin of *S. dysenteriae* while stx2 is a heterogenous group of toxins and more related to Shiga toxin.

The serotype associated with EHEC group is *E. coli* 0157:H7. This group of E. coli starts from mild to severe bloody diarrhea and infection may be asymptomatic or include hemorrhagic colitis, and haemolytic uremic syndrome (HUS), a leading cause of renal failure in children which may be fatal (Wasteson, 2001; Garcia *et al.*,2010). STEC strains of this serotype have been the cause of a numbers of food and waterborne outbreaks and also of numbers of sporadic cases all over the world, since the first hamburger-borne outbreaks were registered in USA in 1992 until 1993 (Wasteson, 2001).

STEC strains have been recovered from faeces or intestines of a wide range of domestics, wild animals and birds (Wasteson, 2001). Cattle have been recognized as the most important vector of the serotype 0157:H7 which then releasing it into the environment and human food chain (Wasteson, 2001; Nielsen *et al.*, 2004). According to Garcia *et al*. (2010), faecal oral route contributes to the transmission of STEC to animals and humans through the contamination of food crops and water sources, and from direct contact.

STEC can be found in animals due to several reasons. In an animal farm, the commercial feed sometimes contaminated with *E. coli* and so with water. According to Wasteson (2001), feed and water represent the most common means of infection. The practise of applying fresh manure to land has been shown to transfer STEC to food crops and water surface (Wasteson, 2001). According to Nielsen *et al.* (2004), there is a possibility that wild animals in close contact with farm animals transmit and act as carriers of STEC.

2.2 Agent of zoonotic diseases

2.2.1 Wildlife reservoirs for zoonoses

Zoonoses are infectious disease that has been transmitted from animals to human (Bengis *et al*., 2004). According to Schaechter *et al.* (1989), the word zoonoses were derived from Greek which "zoon" means animals and "nosos" means disease. Wildlife is known for zoonotic agents as they involved in most of zoonotic diseases (Bengis *et al.*, 2004; Rhyan *et al.*, 2010). Wildlife reservoirs have long been recognized for several important zoonotic diseases, including plague, rabies, and tularemia (Rhyan *et al*., 2010).

An example of zoonotic *E. coli* is enterohemorrhagic *Escherichia coli* (EHEC). According to Garcia *et al*. (2010), the EHEC are illustrative of the One Health concept as they embody the complex ecology of agriculture animals, wildlife, and the environment in zoonotic transmission of EHEC 0157. Transmission of EHEC is via the faecal-oral route and through the consumption of contaminated food and drinks or direct contact with infected animals (Garcia *et al.*, 2010).

According to Bonnedahl (2011), many bird species undergo migration which will creates the potential to establish the new disease along the migration routes. The emergence of West Nile Virus in USA is an example on how the zoonotic disease cause by birds can be dispersed (Bonnedahl, 2011). Opportunities for the emergence of zoonotic diseases depend on the frequency of contacts between wildlife species and humans (Naicker, 2011).

Bats are one of the most widely distributed mammals in the world and they are reservoirs of several zoonoses (Adesiyun *et al.,* 2009). Bats have been proved to cause rabies and SARS virus (Calisher *et al.,* 2008). As they are carriers of pathogenic agents, this makes them important in the epidemiology of bacterial, viral and mycotic zoonoses (Adesiyun *et al.,* 2009). According to Calisher (2006), bats are suitable reservoir for diseases due to the characteristics of bats such as their particular diets, ability to fly, cyclic migration, daily movement patterns and their susceptibility to viruses.

2.2.2 *Escherichia coli* in broiler chicken

According to Wasteson (2001), zoonotic *E. coli* have been recovered from faeces or intestines of a wide range of both domesticated, wild animals and birds. Faecal contamination is one of the contributors to transmissions of pathogenic *E. coli* to human through contamination of food crops, water sources and also direct contact (Wasteson, 2001; Garcia *et al.,* 2010). There are also said that wild animals and birds acts as vehicle for pathogenic *E. coli* in transmission between farms or supporting the infections in domestic animals (Nielson *et al.,* 2004).

E. coli have been isolated from broiler chicken, duck, rodents, bats and many more (Ewers *et al.,* 2009; Adzitey *et al.,* 2012; Nielson *et al.,* 2004; Adesiyun *et al.,* 2009). Jakobsen *et al.* (2010) showed that there are clonal group A (CgA) *E. coli* isolates that are closely related to O15:K52:H1 group has been found in broiler chicken and its meat which may cause Urinary Tract Infections (UTI) to humans. Among the 158 tested isolates, there are 25 CgA isolates were detected from broiler chicken and this indicates that broiler chickens and its meat may be the source of CgA isolates in humans (Jakobsen *et al.,* 2010).

The intestines and environment of the chicken also can be the contributor for transmission of pathogenic *E. coli*. The study done by Ewers and colleagues (2009) indicate that the intestine and environment of chicken can acts as reservoirs for extraintestinal pathogenic *E. coli* strains. Similar to UTI in humans, the chicken intestine can serve as a reservoir for *E. coli* strains capable of causing extraintestinal infections in avian and mammalian hosts which may resulting in potential zoonotic risk (Ewers *et al.,* 2009).

2.3 Pulsed Field Gel Electrophoresis (PFGE)

Pulsed Field Gel Electrophoresis (PFGE) has been proven as the "gold standard" technique which has high discriminatory power (Welinder-Olsson *et al.,* 2002). The conventional agarose electrophoresis cannot resolve large molecules of DNA which is larger than 50bp so this limitation has been overcome by the development of this PFGE (Basim *et al.,* 2001). The new technique of PFGE takes advantage of the elongated and oriented configuration of large DNA molecules in agarose gel at finite field strengths (Basim *et al.,* 2001).

PFGE can be used to compare large genomic DNA fragments after digestion with a restriction enzyme. It can obtain relevant genome size and genetic map of chromosomes of bacteria that are hardly to be determined (Basim *et al.,* 2001). The limit of resolution of PFGE depends on several factors such as uniformity of two electric fields, duration of electric pulses, pulse time between the alternation of field, angles of two electric fields to the gel and ratio of strength of the two electric field (Basim *et al.,* 2001). Based on the protocol of PulseNet, the preparation of DNA for PFGE starts with lysing the bacteria that are encased in an agarose plug. After multiple wash, DNA within the agarose is digested with restriction enzymes and electrophoresed by using PFGE.

Compared to the classical electrophoresis, PFGE involves periodical changing of field direction so the various length of DNA react to the change at differing rates and the larger DNA fragments will be slower to realign their charge when field direction is changed. Classical electrophoresis uses single electric field which causes the DNA molecules to migrate according to the mass which cause the larger DNA fragment block in the upper part as it cannot pass through. According to Basim *et al.* (2001), PFGE has shown excellent ability to separate small, natural linear chromosomal DNAs ranging in size from 50kb parasite microchromosomes to multimillion-bp yeast chromosomes.

The PFGE method has been commonly used for typing of number of bacterial species and outbreak investigations (Durmaz *et al.,* 2009; Rounds *et al.,* 2010; Zou *et al.,* 2010). PFGE has been used widely in the study of *E. coli* strains. For example, *E. coli* 0157:H7 was detected in undercooked hamburger meat (Barrett *et al.,* 1994), marketed beef (Apun *et al.*, 2006) and the epidemiological relationship of isolates of *E. coli* causing

edema disease (Aarestrup *et al.,* 1997) has been analyzed by pulsed-field gel electrophoresis (PFGE). Besides that, PFGE also has been used in the study of other strains type such as *Salmonella* spp. (Cardinale *et al.,* 2005; Zou *et al.,* 2010; Rounds *et al.,* 2010) and *Bordetella pertussis* (Advani *et al.,* 2004).

The PFGE method is often referred as "gold standard" method due to its high discriminatory power. This statement has been proven by Welinder-Olsson *et al.,* (2002) and study done by Apun *et al.* (2006) which PFGE showed a good ability to discriminate between *E. coli* isolates of serogroup 0157:H7 and 0157, STEC and other *E. coli* isolated from beef market in East Malaysia. The high discriminatory ability of PFGE analysis was clearly confirmed by the study.

2.3.1 PFGE technique

The PFGE technique basically consist of 5 steps which are plug preparation, cell lysis, washing of plug, DNA restriction and electrophoresis of restricted DNA fragments (PulseNet, 2009). Researchers use their own procedures for the PFGE steps as an optimized standard PFGE protocol for bacteria typing has not been defined yet (Durmaz *et al.,* 2009). In this study, the protocol from PulseNet, USA (2009) was used with several modifications.

For the plug preparation, the OD concentration of *E. coli* DNA was adjusted to 1.2-1.4 by using spectrophotometer to wavelength of 610 nm. The OD concentration was higher than the one given in the protocol. This is because the protocol before has been used

in a study and it is said that the PFGE profiles obtained were not clear. Adjusting the cell concentration is critical point and after several test, the best resolution obtained is that with a concentration of 1.0 absorbance at 590nm wavelength (Durmaz *et al.,* 2009).

Restriction enzyme, also referred as restriction endonuclease will cleaves double stranded DNA at specific site. Each RE has specific recognition site and requirement to achieve optimal activity. In this study, *Xba*I was used since it has been proven to yield about 20 restriction bands and have been most reproducible on chromosomal DNA of *E. coli* and *Klebsiella* spp. (Mamlouk *et al.,* 2006; Apun *et al.,* 2006). There were 2 ways provided in conducting the restriction digestion of the *E. coli* DNA which were quick RE and slow RE. After conducting these experiment, the slow RE technique gives a clear banding pattern compared to quick RE technique. So, it can be concluded that the 16 hours incubation under appropriate amount of RE master mix gives better PFGE profile.

MATERIALS AND METHODS

3.1 Samples collection and enrichment

3.1.1 Source of sample

The sample of *Escherichia coli* (*E. coli*) were taken from microbiology postgraduate student's stock culture collections. There were a total of 22 *E. coli* isolate. For bats, there were 2 isolates from Matang Wildlife Centre, 4 isolates from Kubah National Park, 3 isolates from Nanga Merit Kapit and 2 isolates from Sibu whereas there were 6 isolates of broiler chicken and 5 isolates from soil collected under the chicken coop as shown in Table 1 and 2. The samples were chosen based on different habitat in order to study the genetic diversity of the isolates.

Table 1: *E. coli* isolated from bats

Habitats	Sample no.	Common name	Scientific name
Bukit Aup Sibu	C0813(A)	Short nosed fruit bats	*Cynopterus brachyotis*
	C0818(A)	Short nosed fruit bats	*Cynopterus brachyotis*
Nanga Merit Kapit	FNM133(I)	Fawn roundleaf bats	*Hipposideros cervinus*
	FNM015(I)	Short nosed fruit bats	*Cynopterus brachyotis*
	U2037(A)	Short nosed fruit bats	*Cynopterus brachyotis*
Matang Wildlife Centre	TK172776(F)	Short nosed fruit bats	*Cynopterus brachyotis*
	TK172787(F)	Fawn roundleaf bats	*Hipposideros cervinus*
Kubah National Park	TK172792(A)	Dayak roundleaf bats	*Hippsoderos dyacorum*
	TK172792(F)	Dayak roundleaf bats	*Hippsoderos dyacorum*
	TK172822(A)	Hardwicke's wolly bats	*Kerivoula hardwicki*
	TK172823(A)	Hardwicke's wolly bats	*Kerivoula hardwicki*

Table 2: *E. coli* isolated from broiler chicken and soil

Habitats	Sample no.	Type of sample
Kuching	A806(I)	Broiler chicken
	B0806(I)	Broiler chicken
	A1506(I)	Broiler chicken
	B1506(I)	Broiler chicken
	A2206(I)	Broiler chicken
	B2206(I)	Broiler chicken
	S14A(I)	Soil under the chicken coop
	S14B(I)	Soil under the chicken coop
	S21A(I)	Soil under the chicken coop
	S21B(I)	Soil under the chicken coop
	S28A(I)	Soil under the chicken coop

3.1.2 Enrichment of samples

The samples from culture collection were stored in glycerol stock and Nutrient Agar (NA) slant. The bacteria from glycerol stock then were cultured in LB broth (Difco, USA) and incubated for 24 hours for enrichment before streak onto EMB (Oxoid, England) agar. While the bacteria from NA slant had been streak onto EMB (Oxoid, England) agar. Then, the EMB agar was incubated overnight at 37^{o}C. EMB agar is a selective medium for *E. coli* which will exhibit a green metallic sheen by reflected light. Thus, the single colonies of green metallic sheen that were produced were then sub-cultured and stored in NA slant.

3.1.3 Bacterial Culture

The *E. coli* from each isolates was streak onto Nutrient Agar (Oxoid, England) plate for confluent growth. Then, the bacterial culture was incubated at 37ºC for overnight.

3.2 PFGE Techniques

The PFGE technique that were used in this study was adapted from PulseNet USA, 2009 with some modification

3.2.1 Preparation of DNA

Cell Suspension Buffer (CSB) (100 mM Tris: 100 mM EDTA, pH 8.0) was prepared at 3 ml in each of the universal bottles. The overnight growth bacteria from NA plate then was transferred into CSB in the universal bottles. Then, the concentration of the cell suspension was adjusted using spectrophotometer to wavelength of 610 nm and absorbance (Optical Density) of 1.2-1.4.

3.2.2 Casting plug

The adjusted cell suspension was transferred into labelled 1.5 ml microcentrifuge tubes and Proteinase K (Promega, USA) (20 mg/ml) was added to each of the tubes. Then, melted 1% Low Melting Point (0.375g LMP in 25 ml TE buffer) (GibcoBRL, USA) agar was added to the cell suspension. The suspension then was dispensed to a plug molds (Bio-Rad) by using 200 µl pipette and allowed to solidify at room temperature for 10-15 minutes.

3.2.3 Lysis of cells in agarose plugs

Cell Lysis Buffer (CLB) (50 mM Tris: 50 mM EDTA, pH 8.0, 1% Sarcosyl) was prepared at 5 ml in each universal bottles. Then, 25 µl of Proteinase K (20 mg/ml) was added into each of the CLB. The plugs then was transferred into each labelled universal bottles that contained CLB and was incubated in a 55ºC shaker water bath for 2 hours with constant and vigorous agitation (100 rpm).

3.2.4 Washing of Agarose Plugs

The lysis buffer in the bottle then was carefully removed by using 5 ml pipette and 15 ml of sterile Ultrapure water (CLRW) that was pre-heated at 55ºC was added to each bottles. After that, the bottles were incubated at 55ºC shaker water bath for 15 minutes with constant and vigorous agitation at 100 rpm. The washing step with ultrapure water was repeated for one more time. Then, the washing step was continued by using pre-heated TE buffer. After removed the sterile ultrapure water, 15 ml of pre-heated sterile TE buffer was added to each of the bottles and incubated for 15 minutes in the shaker water bath. The washing step with TE buffer was repeated for three times. Lastly, the plugs was stored in the universal bottles with 5 ml TE buffer and stored in 4°C until RE digestion.

3.2.5 Restriction digestion of DNA in Agarose Plugs

The restriction enzyme *XbaI* (Promega, USA) was used to digest the DNA for each isolate. *Xba*I is a restriction endonuclease which cut at 5'-TCTAGA-3'. There were two ways to do the restriction digestion which are quick RE and slow RE. For quick RE, pre- restriction master mix (Promega, USA) was prepared and 200 µl of the solution buffer was

transferred into each labelled microcentrifuge tubes. The plugs then were carefully being removed from the TE buffer and were cut at size 2.0-2.5mm. After that, the plugs slices were transferred into pre-restriction solution buffer in the tubes and were incubated in 37ºC water bath for 10 minutes. After the incubation, the buffer was carefully being removed by using 200 µl pipette and 200 µl of restriction enzyme solution was added. The plugs then will be incubated in 37ºC water bath for 2 hours.

Table 3: Pre-restriction master mix (Quick RE)

Reagent	µl/ Plug Slice	µl/ 15 Plug Size
Sterile Clinical Laboratory Reagent Water	178µl	2670 µl
Restriction Buffer	20µl	300 µl
BSA	2 µl	30 µl
Total Volume	200 µl	3000 µl

Table 4: Restriction enzyme master mix (Quick RE)

Reagent	µl/ Plug Slice	µl/ 15 Plug Size
Sterile Clinical Laboratory Reagent Water	173 µl	2595 µl
Restriction Buffer	20 µl	300 µl
BSA	2 µl	30 µl
Xba1	5 µl	75 µl
Total Volume	200 µl	3000 µl

For slow RE, pre-restriction master mix (Promega, USA) was prepared at 50 µl for each tube. Then, the pre-RE solution were added into each plugs slices and were incubated on ice for 20-30 min. After removed the pre-RE mixture, 50 µl of RE master mix were added into each tube and incubated on ice again for 15 min. Lastly, the tube with plug slices were incubated in 37°C water bath for 16-16.5 hours.